A Dissertation for the Degree of Doctor of Philosophy in Pharmacy

Discovery of Novel Leucyl-tRNA Synthetase (LRS)-Targeted Mammalian Target of Rapamycin Complex 1 (mTORC1) Inhibitors

[Part 1] Discovery of (*S*)-Isobutyloxazolidin-2-one as a Novel LRS-targeted mTORC1 Inhibitors

[Part 2-1] Discovery of Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

[Part 2-2] Structure Activity Relationship (SAR) Studies of Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

[Part 3] Discovery of Simplified Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

[Part 4] Discovery of New Series of Simplified Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

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Abstract

Discovery of Novel Leucyl-tRNA Synthetase (LRS)-Targeted Mammalian Target of Rapamycin Complex 1 (mTORC1) Inhibitors

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Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that incorporates both intracellular and extracellular signals and plays a crucial role in cell metabolism, growth, proliferation and autophagy. mTOR exists in two structurally and functionally different multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). In particular, overactive mTORC1 reported to be associated with various human diseases such as diabetes, neurodegeneration and cancers.

Rapamycin and its analogs which are well known allosteric inhibitor of mTORC1, binds to the FK506-binding protein 12 (FKBP12) and interacts with the FKBP12-rapamycin binding (FRB) domain in mTORC1. Although rapamycin and its analogues have been developed for the use of anti-cancer agents, these agents have limitations that only partially inhibit mTORC1 activity and rapamycin alone is not sufficient to control the mTORC1 activity in many pathological conditions. Thus, small molecules targeting other

possible regulators can offer alternative strategy to overcome rapamycin resistance in anti-cancer agents.

Leucyl-tRNA synthetase (LRS) has been reported to be a possible mediator of intracellular amino acids signaling to mTORC1. LRS is a member of the class I aminoacyl-tRNA synthetase (ARSs) family that catalyzes the ATP-dependent ligation of leucine to cognate tRNA in protein biosynthesis. Recent studies indicate that LRS may act as a leucine sensor for the mTORC1 pathway. Given that mTORC1 is associated with cell proliferation and tumorigenesis, the LRS-mediated mTORC1 pathway may offer an alternative strategy in anticancer therapy.

We have developed one of the leucinol analogs, (*S*)-4-isobutyloxazolidine-2-one as a LRS-targeted mTORC1 inhibitors. This compound inhibited downstream phosphorylation of mTORC1 by blocking leucine-sensing ability of LRS, without affecting the catalytic activity of LRS. In addition, it exhibited cytotoxicity against rapamycin-resistant colon cancer cells, suggesting that LRS has the potential to serve as a novel therapeutic target.

Next, we developed leucyladenylate sulfamate derivatives as LRS-targeted mTORC1 inhibitors. We demonstrated that (*S*)-2-hydroxy-4-methylpentanoyl adenylate sulfamate selectively inhibited LRS-mediated mTORC1 activation and exerted specific cytotoxicity against colon cancer cells with a hyperactive mTORC1.

Furthermore, we replaced the adenylate group with a *N*-(3,4-dimethoxybenzyl)benzenesulfonamide or a *N*-(2-phenoxyethyl)benzenesulfonamide groups that can maintain specific binding, but has more favorable physicochemical

properties such as reduced polarity and asymmetric centers. Among these simplified analogues, we discovered that the compound and its constrained analogue effectively inhibited S6K phosphorylation in a dose-dependent manner and exhibited cancer cell specific cytotoxicity against six different types of cancer cells.

In our continuing efforts to expand our in-house library of simplified leucyladenylates analogues, we decided to design new scaffolds based on the gefitinib structure. We devised new series of simplified structures by introducing *N*-(3-chloro-4-fluorophenyl) quinazolin-4-amine instead of adenine group and various linker structures to replace 5-*O*-sulfamoylribose. We demonstrated that compounds with sulfonamide, ethyl alcohol or ethyl amino linker in new series of simplified adenylate structure showed potent inhibition. Furthermore, these compounds showed general cytotoxicity against various types of cancer cell lines, suggesting that they have a potential as effective anticancer agent.

Keywords: Leucyl-tRNA synthetase, mTORC1 Inhibitor, Leucinol, Anticancer agent

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Table of Contents

List of Schemes

List of Figures

List of Tables

I. Introduction

1. Mammalian target of rapamycin (mTOR)

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that incorporates both intracellular and extracellular signals and plays a crucial role in cellular metabolism, growth, proliferation and autophagy. mTOR exists in two structurally and functionally different multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) which have different sensitivities to rapamycin as well as upstream inputs and downstream outputs. mTORC1 regulates protein synthesis by phosphorylating downstream two major substrates, S6 kinase 1 (S6K1) and the translational regulators eukaryotic translation initiation factor 4E (elF4E)-binding protein 1 (4E-BP1), whereas mTORC2 regulates cytoskeletal organization, cell survival and metabolism.[1] Given that the signaling pathways involving S6K1 and 4E-BP1 have been linked to tumorigenesis in many tissues, [2-4] rapamycin and its analogues have been developed for the use of anti-cancer agents.[5,6]

mTORC1 is composed of six different protein subunits whereas mTORC2 is composed of seven protein components. They share the catalytic mTOR subunits, mammalian lethal with sec13 protein 8 (mLST8, also known as GβL), DEP domain containing mTOR-interacting protein (DEPTOR) and the Tti1/Tel2 complex. In contrast, regulatory-associated protein of mammalian target of rapamycin (Raptor) and proline rich Akt substrate 40 kDa (PRAS40) are specific to mTORC1, whereas rapamycin-insensitive

companion of mTOR (Rictor), mammalian stress-activated map kinase-interacting protein 1 and 2 (Protor 1/2) are specific to mTORC2.[1]

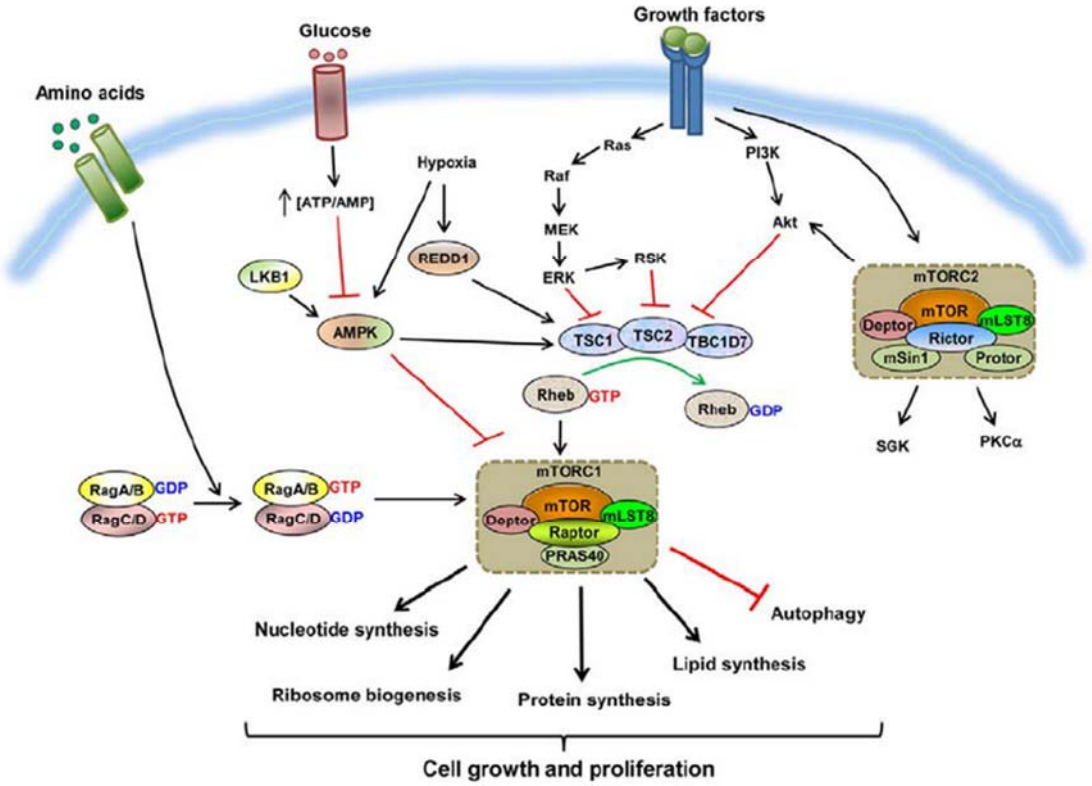


Figure 1. mTOR signaling pathway.[7]

2. mTORC1 inhibitors

The most studied compounds are rapamycin and its derivatives that directly bind near the mTOR kinase domain by forming a complex with the FK506-binding protein 12 (FKBP12); temsirolimus and everolimus have been approved by the FDA for the treatment of advanced renal cell carcinoma,[5,6] and ridaforolimus showed a promising

outcome in a recent clinical trial for advanced soft tissue and bone sarcoma.[8,9] Unfortunately, the efficacy of these rapamycin analogues, also called rapalogs, for the treatment of various cancer patients has been generally disappointing, mainly because these agents are cytostatic and only partially inhibit mTORC1 activity.[10,11] Although rapamycin is a highly specific allosteric inhibitor of mTORC1, the mTORC1 pathway involves multiple regulatory mechanisms and complex feedback loops that have not yet been fully understood, thus resulting in an incomplete inhibition of kinase activity. Therefore, small molecules targeting other possible regulators may offer an alternative strategy for the suppression of mTORC1 activity, and have the potential to overcome rapamycin-resistance in cancer treatment.

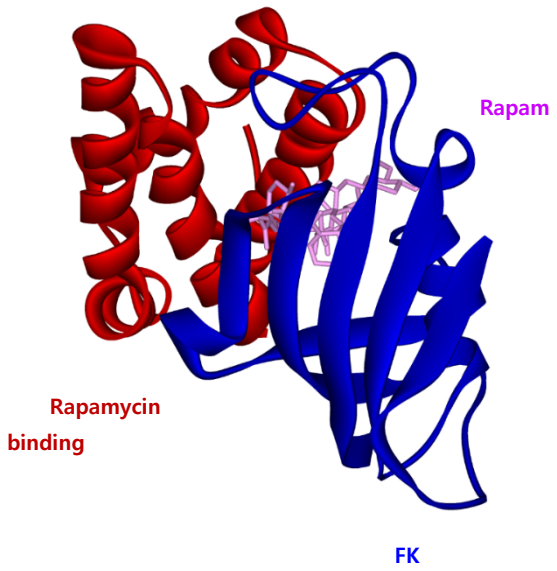


Figure 2. Ribbon diagram of human FKBP12 in complex with rapamycin interacting with the rapamycin domain of mTOR.

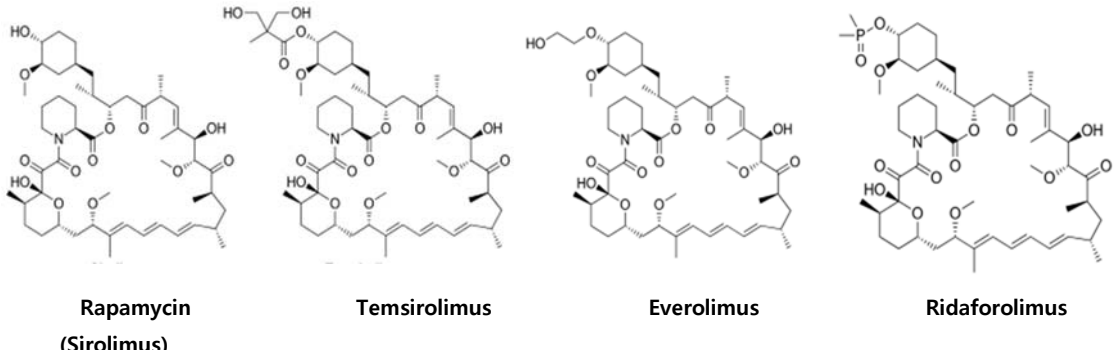


Figure 3. Structure of rapamycin and its analogues.

3. LRS as a leucine sensor

mTORC1 activity is regulated by various environmental signals, such as the levels of nutrients, energy, and oxygen. Although the complete mechanism of how mTORC1 senses these signals still remains a mystery, the proteinogenic amino acid leucine is considered to be the master controller in amino acid-dependent mTORC1 signaling.[12,13] More importantly, several recent studies have reported that leucyl-tRNA synthetase (LRS) may act as an intracellular leucine sensor by directly binding to RagD GTPase, one of the key mediators of the amino acid-dependent mTORC1 pathway.[14,15] LRS is a member of the class I aminoacyl-tRNA synthetase (ARSs) family that catalyzes the ATP-dependent ligation of amino acids to cognate transfer RNA (tRNA) in protein biosynthesis. Because of their pivotal role in cell survival, ARSs have been effective targets for antibiotics to overcome bacterial resistance,[16-19] and also have been studied as anti-cancer agents.[20-22] In addition to its traditional role, LRS appears to participate in mTORC1 activation by acting as a GTPase-activating protein (GAP) for Rag GTPase in a leucine-dependent manner. Moreover, the leucine-induced activation of mTORC1 can be inhibited by

leucine analogues, such as leucinol, without affecting the leucine charging ability of LRS, suggesting that LRS-targeted inhibitors can suppress mTORC1 activity.[14, 23, 24]

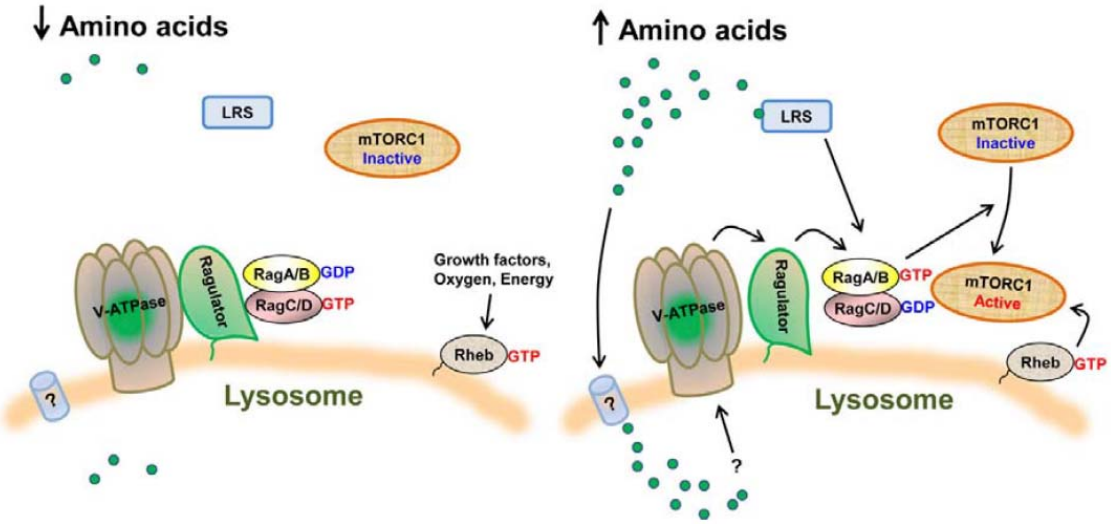


Figure 4. Amino acid signaling to mTORC1.[7]

4. Strategic Approach

It is known that LRS functions as an intracellular leucine sensor in the amino acid-dependent mTORC1 pathway by its activity as a GTPase-activating protein (GAP) for RagD.[14] It is found that LRS directly interacts with GTP bound form of RagD GTPase but not with RagA, RagB and RagC. LRS interacts with only RagB/RagD heterodimers and forms a molecular complex with RagD and Raptor in an amino acid-dependent manner. When leucine concentration increases in the cell, lysosomal LRS acts as a leucine sensor and binds to GTP bound form of RagD and facilitates the conversion to an active form, leading to the lysosomal localization and activation of mTORC1.

As a new strategic approach, we designed the novel LRS-targeted mTORC1 inhibitor that can directly binds to LRS to inhibit mTORC1 signaling pathway. As our inhibitor binds to LRS catalytic site, the conformational change of LRS will prevent binding of LRS with RagB/RagD heterodimers to inhibit mTORC1 activation.

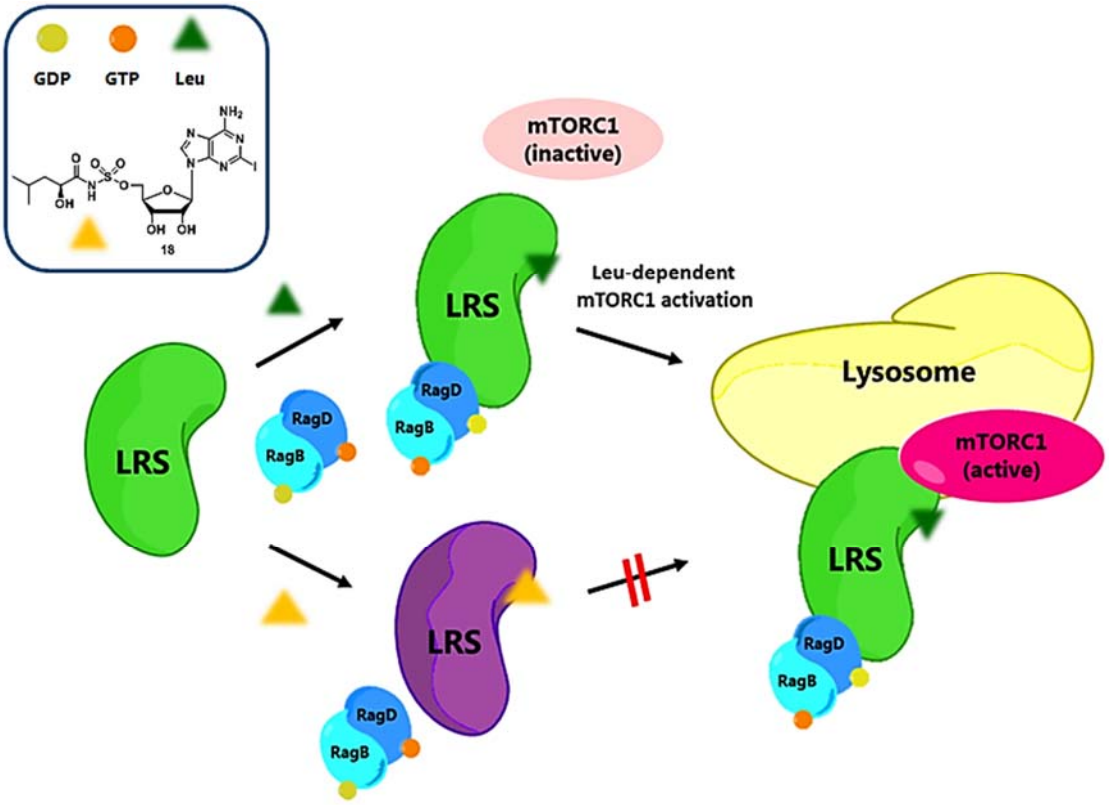


Figure 5. Mechanism of novel LRS-targeted mTORC1inhibitors.

II. Part 1. Discovery of (*s*)-Isobutyloxazolidin-2-one as a Novel LRS-targeted mTORC1 Inhibitors

1. Design background & strategy

To develop LRS-targeted mTORC1 inhibitors, we first designed and synthesized a series of leucinol analogues.[25] It has been reported that leucinol, which can be synthesized from the reduction of the carboxylic acid group of leucine, can block the amino acid stimulation of TOR signaling.[23] Based on the leucinol structure, we designed the leucinol analogues by modifying the hydroxyl group of leucinol and also introduced cyclic structures (**Figure 6**).

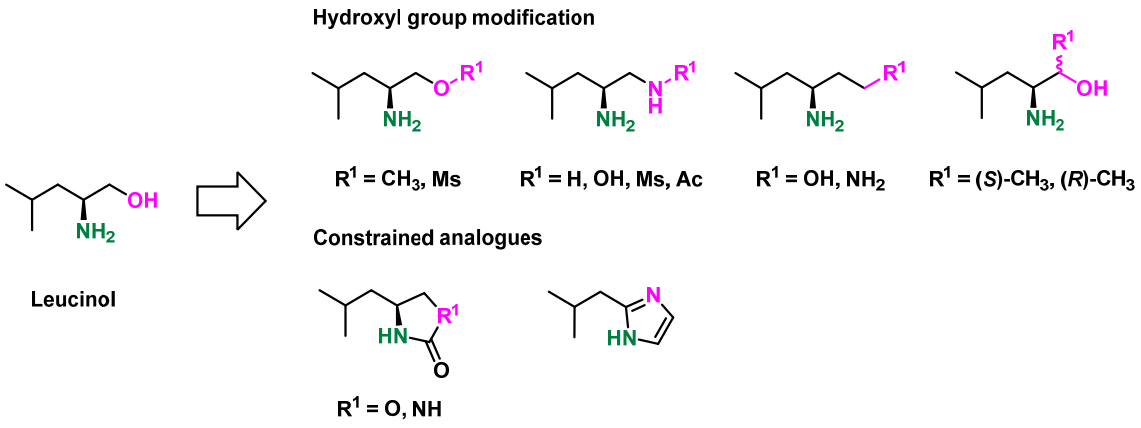
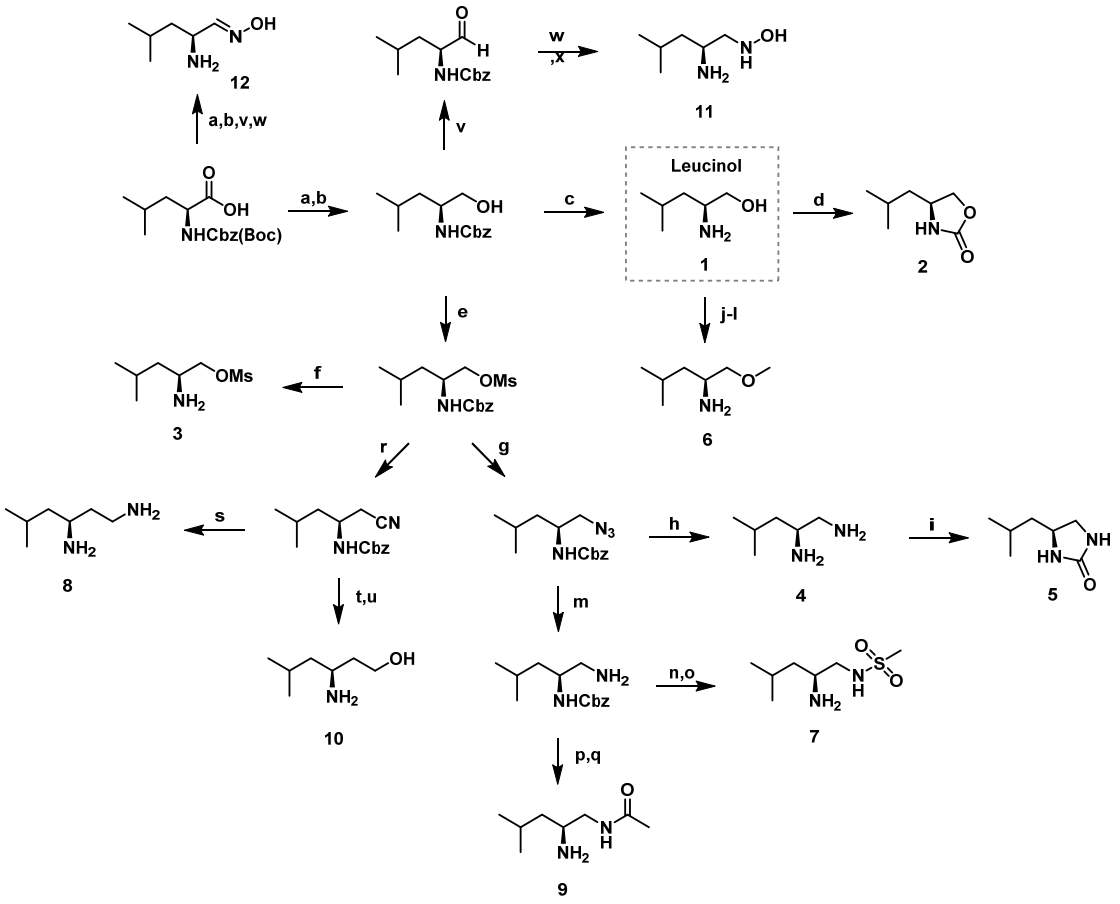


Figure 6. Leucinol and its analogues.

2. Result and Discussion

2.1. Chemistry

Leucinol **1** and compounds **2**-**12** were prepared by following the pathway described in **Scheme 1**. Compounds **2** and **6** were prepared by carbonylation or methylation from leucinol respectively, which was prepared from commercially available *N*-Cbz-leucine by 3 steps. Compounds **3**, **8**, and **10** were synthesized from the mesylated product of *N*-Cbz-leucinol; compound **3** was obtained via deprotection of the *N*-Cbz group, and compounds **8** and **10** were prepared by displacing the mesylate with a nitrile group followed by reduction or reductive hydrolysis respectively. Displacement of the mesylate with sodium azide yielded an azido intermediate, which was then used to prepare compounds **4**, **5**, **7**, and **9**. Hydrogenation in the presence of 10% palladium afforded diamine compound **4**, and carbonylation of compound **4** yielded a cyclic analog, compound **5**; selective reduction of the azide produced the *N*-Cbz protected monoamine intermediate, and subsequent methanesulfonylation or acylation, followed by deprotection produced compound **7** and **9** respectively. To prepare compound **11**, *N*-Cbz protected leucinol was oxidized to aldehyde by Dess-Martin periodinane, and subsequent condensation with hydroxylamine, followed by deprotection yielded compound **11**. Compound **12** was prepared from *N*-Boc protected leucine by applying the same procedure carried out for compound **11.**

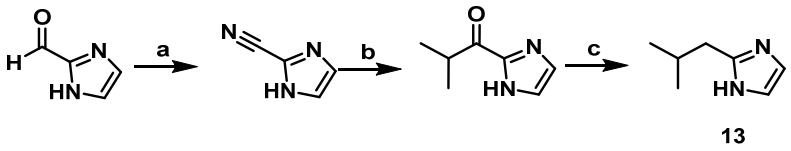


Scheme 1. Synthesis of leucinol analogures **1**-**12**.

Reagents & conditions: (a) cat. *conc*. H_2SO_4, MeOH, reflux, 12 h, 99% for *N*-Cbz, 45% for *N*-Boc; (b) $NaBH_4$, EtOH, 0 °C→r.t, 56% for *N*-Cbz, 55% for *N*-Boc; (c) Pd/C, H_2, MeOH, r.t, overnight, 89%; (d) 1, 1'-carbonyldiimidazole, DMF, r.t, 12 h, 39%; (e) MsCl, TEA, MC, 0 °C→r.t, 2 h, 90%; (f) 10% Pd/C, H_2, MeOH, r.t, 12 h, 89%; (g) NaN_3, DMF, 80 °C, 4 h, 78%; (h) 10% Pd/C, H_2, MeOH, r.t, 12 h, 33%; (i) 1,1'-carbonyldiimidazole, MC, r.t, 12 h, 54%; (j) NaH, MeI, DMF, 0 °C→r.t, 12 h; (k) benzyl chloroformate, K_2CO_3, acetonitrile, 0 °C→r.t, 5 h, 65%; (l) 10% Pd/C, H_2, 2M NH_3 in MeOH, r.t, 12 h, 99%; (m) PPh_3, cat. H_2O, THF, r.t, 12 h, 83%; (n) MsCl, TEA, MC, r.t, 1 h, 89%; (o) 10% Pd/C, H_2, 2M NH_3 in MeOH, r.t, 12 h, 79%; (p) acetic anhydride, 80 °C, 12 h, 99%; (q) 10% Pd/C, H_2, 2M NH_3 in MeOH, r.t, 12 h, 98%; (r) NaCN, 15-crown-5, DMF, 60 °C, 48 h, 73%; (s) 10% Pd/C, H_2, MeOH, r.t, 12 h, 70%; (t) 6N HCl, reflux, 12 h, 99%; (u) BH_3·THF, THF, reflux, overnight, 15%; (v) Dess-Martin periodinane, MC, r.t, 2 h, 61% for N-Cbz, 80% for N-Boc; (w) hydroxylamine hydrochloride, Na_2CO_3, MeOH, r.t, 12 h, 66% for *N*-Cbz, 67% for *N*-Boc; (x) 10% Pd/C, H_2, MeOH, r.t, 12 h, 86%.

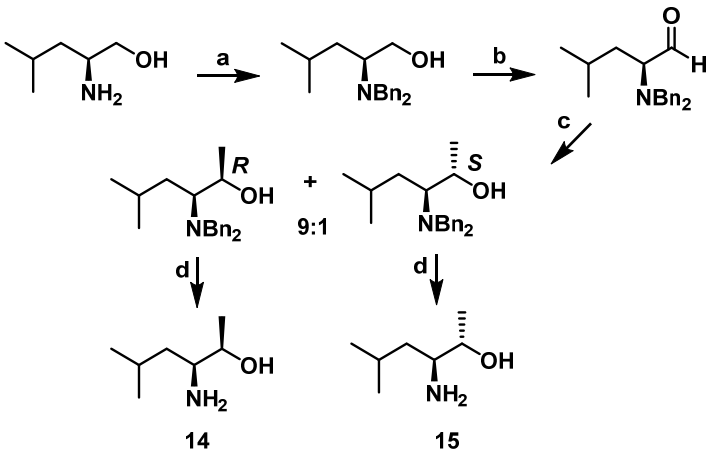
Compound **13** was synthesized from commercially available imidazole-2-carboxaldehyde (**Scheme 2**); the aldehyde group was converted to a nitrile group which was then reacted with isopropyl magnesium chloride to produce an imidazole intermediate containing an isobutyl ketone group. The ketone group was completely reduced by using Ni-Al catalyst to generate compound **13**.

Leucinol analogs with a chiral a-methyl group were synthesized by following the pathway described in **Scheme 3**. *N*-Dibenzyl protected leucinol was oxidized to aldehyde for subsequent Grignard reaction, providing a racemic alcohol mixture of *R*- and *S*- isomers with a ratio of 9:1. Final debenzylation of purified enantiomers yielded compounds **14** (*R*-isomer) and **15** (*S*-isomer). The stereochemistry of each compound was assigned based on the previous report.[26]



Scheme 2. Synthesis of compound **13**.

Reagents & conditions: (a) i) hydroxylamine hydrochloride, pyridine, r.t, 2 h, ii) acetic anhydride, 80 °C→r.t, 5 h, 80%; (b) isopropylmagnesium chloride, THF, 0 °C→r.t, 3 h, 77%; (c) Ni-Al alloy, H_2O, reflux, 48 h, 50%.



Scheme 3. Synthesis of compounds **14** and **15**.

Reagents & conditions: (a) benzyl bromide, K_2CO_3, aq. MeOH, 65 ºC, 2 h, 41%; (b) oxalyl chloride, DMSO, TEA, MC, -78 ºC, 2 h; (c) CH_3MgI, ether, 0 ºC→r.t, 3 h, 60% (*R*:*S*=9:1); (d) 10% Pd/C, H_2, MeOH. r.t, overnight, 62% for **14**, 47% for **15**.

2.2. Biological Activity

As a primary screening, we first assessed the effect of each compound on leucine-induced phosphorylation of S6 Kinase (S6K) by immunoblotting. S6K is one of the mTORC1 substrates, and pretreatment with leucinol appears to block phosphorylation of S6K by inhibiting LRS.[16] We treated HEK293 cells with each compound at 1 mM concentration, along with rapamycin as a control at 100 nM; and then activated mTORC1 by treating cells with leucine for 10 min. As demonstrated in **Figure 7**, leucine treatment induces phosphorylation of S6K, whereas pretreatment with rapamycin or deprivation of leucine blocked the phosphorylation. Pretreatment of leucinol also inhibited the phosphorylation to some extent, while compounds, **2, 5, 11**, and **13** appeared to inhibit the phosphorylation more effectively than leucinol at the same concentrations. It is interesting to note that all four compounds have multiple heteroatoms that can participate in additional hydrogen

bond interactions compared to leucinol. It is also noticeable that compounds **2**, **5**, and **13** are all cyclic analogs. Introduction of an additional methylene group or chiral a-methyl group did not generate any desirable activity. Addition of a relatively bulky functional group such as methanesulfonyl, mesyl, and acetyl group at the alcohol position also do not produce inhibitory effects, suggesting that leucine binding site is highly specific and only afford a small sized residue.

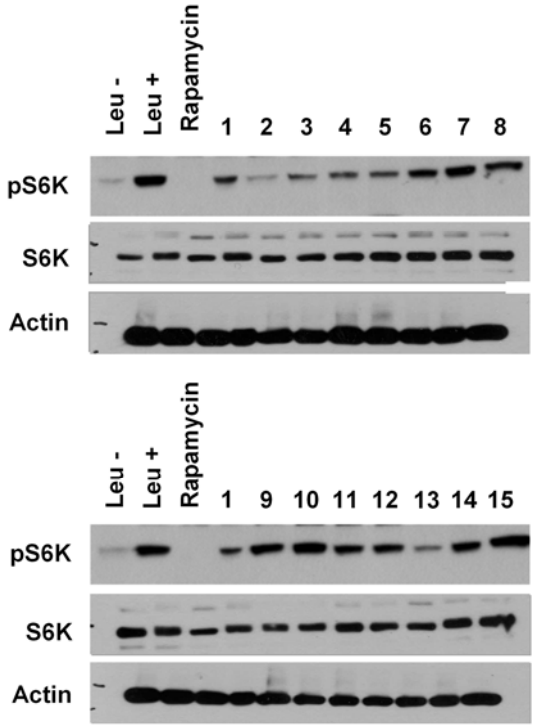


Figure 7. Inhibition of LRS-mediated mTORC1 activation of synthesized leucinol analogues on HEK293 cells.

Based on the primary screening results, we decided to focus on compounds **2**, **5**, **11**, and **13**, and further determined the inhibitory effects on leucine-induced mTORC1 activation at the various concentrations by immunoblotting. As shown in **Figure 8**, all four compounds inhibited S6K phosphorylation in a dose-dependent manner while their

apprent potency decreases in the order of **5** > **13** > **11** > **2**. The cyclic analogs, compounds **5** and **13** appear to be more potent than leucinol, however, another cyclic derivative compound **2** only differs from compound **5** by one atom, and yet exhibits significantly lower activity. Additionally, we examined the effect of each compound on mTORC2 activation by using Akt as the marker.[1] All tested compounds including rapamycin and leucinol do not affect the cellular levels of Akt or phosphorylated Akt (pAkt) indicating that biological effects of these compounds are indeed mTORC1 specific.

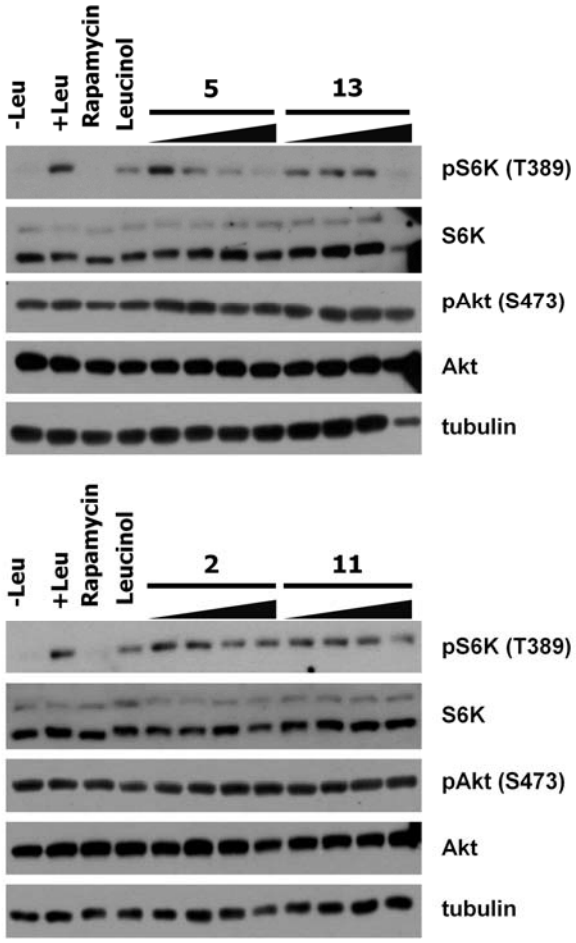


Figure 8. Dose-dependent inhibition of LRS-mediated mTORC1 activation of selected leucinol derivatives on HEK293 cells. Rapamycin was treated at 100 nM, leucinol was at 800 μM, and compounds 2, 5, 11, and 13 were treated at 100, 200, 400, and 800 μM.

To investigate the mechanisms of leucine analogs in LRS-mediated mTORC1 activation, we examined the effects of compounds **5** and **13** on the catalytic activity of LRS. More specifically, since LRS catalyzes leucylation in the presence of tRNA, we carried out leucylation assays on these compounds. As shown in **Figure 9**, both compounds **5** and **13** are poor inhibitors of aminoleucylation, having IC_{50} values in the high millimolar range. Given that both compounds inhibited phosphorylation of S6K at micromolar concentrations, our result indicates that these leucine analogs do not affect catalytic activity of LRS, which is in agreement with the previously reported observation that LRS activates mTORC1 signaling via leucine recognition without involving tRNA charging.[14]

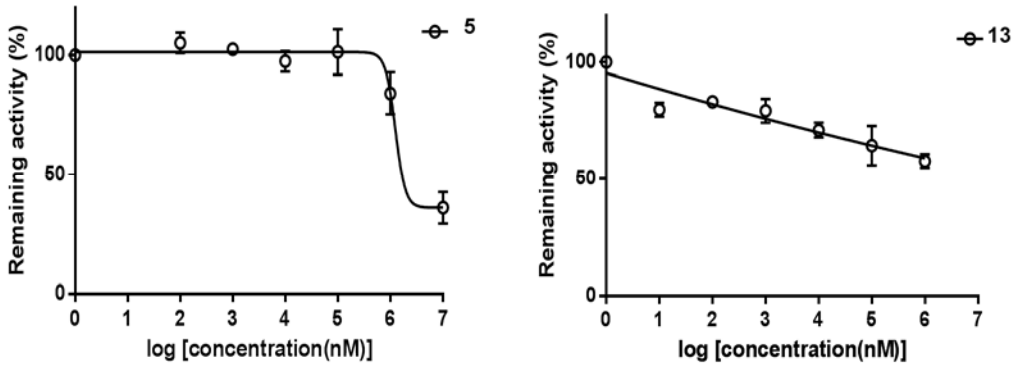


Figure 9. Aminoacylation activity of compound **5** and **13**.

Since mTORC1 is found to be overactive in many cancers, we evaluated cytotoxicity of compound **5** in cancer cells. In particular, mTORC1 is reported to be hyperactive in human colorectal cancer,[27] however, rapamycin appears to be mostly ineffective.[28] We

selected a human colon cancer cell line (SW620) which is known to be rapamycin resistant,[29] to see whether blocking LRS can solve the resistance issue. After treating cells with each compound, we performed cytotoxicity assays by using CellTox Green fluorescent dye which stains the DNA from dead cells (**Figure 10**). As we expected, rapamycin did not exhibit significant cytotoxicity while leucinol seemed to be marginally cytotoxic even at the high concentrations; however, compound **5** demonstrated a dose-dependent cytotoxicity, exerting a much greater magnitude of cytotoxic effects at the same concentrations compared to leucinol. Considering that compound **5** was more potent inhibitor of mTORC1 than leucinol, cytotoxicity of compound **5** could be attributed to mTORC1 inhibition.

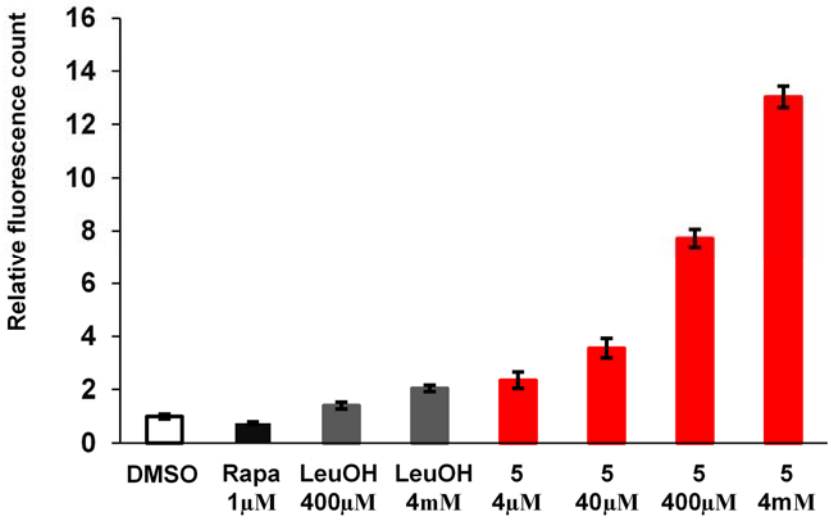


Figure 10. Cytotoxicity of compounds **5** in rapamycin-resistant colon cancer cell (SW620)**.**

3. Conclusion

In summary, we have developed a series of leucinol analogs that can inhibit mTORC1 signaling pathway by blocking leucine-sensing ability of LRS. These analogs act as a competitive inhibitor of leucine in LRS-mediated activation of mTORC1, thereby inhibiting downstream phopshoprylation of S6K. *In vitro* mTORC1 inhibition study showed that compound **5**, which contains an imidazolidin-2-one side chain, effectively inhibited leucine-mediated mTORC1 activation; however compound **5** did not suppress mTORC2 activation, nor did it affect catalytic activity of LRS. Furthermore, compound **5** exhibited much higher cytotoxicity than leucinol against rapamycin-resistant colon cancer cells, implying that effective inhibition of leucine-sensing in the mTORC1 pathway may play an important role in conferring cytotoxicity. Taken together, we believe that the blockade of leucine-sensing ability of LRS in the mTORC1 pathway has the potential to serve as a novel therapeutic target.

III. Part 2-1. Discovery of Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

1. Design background & strategy

Previously, we have demonstrated that a leucine analog, (*S*)-4-isobutyloxazolidin-2-one, selectively inhibited downstream phosphorylation of mTORC1 by blocking the leucine-sensing ability of LRS.[25] Furthermore, this analog exhibited cytotoxicity against rapamycin-resistant colon cancer cells without affecting the catalytic activity of LRS, indicating that LRS has the potential to serve as a novel therapeutic target. In a continuing effort to develop LRS-targeted mTORC1 inhibitors, we designed a new series of compounds based on the structure of aminoacyl adenylate **1**, an enzymatic reaction intermediate of ARSs.[30] Aminoacyl adenylates have been extensively studied as a lead compound for ARS inhibitors.[31,32] Specifically, replacing the hydrolytically unstable acylphosphate group with its isosteres, such as acylsulfamate, resulted in chemically stable and potent inhibitors with IC_{50} values in the submicromolar to low micromolar range versus the corresponding ARSs.[33-35] Based on these previous findings, we synthesized a library of leucyladenylate sulfamates by modifying adenine, ribose and leucine moieties and evaluated their biological activity. Among the more than 70 compounds that we studied, herein we describe three representative compounds shown in **Figure 11**. Each of these three compounds contains distinct structural features that are required for either LRS inhibition or LRS-mediated mTORC1 suppression.

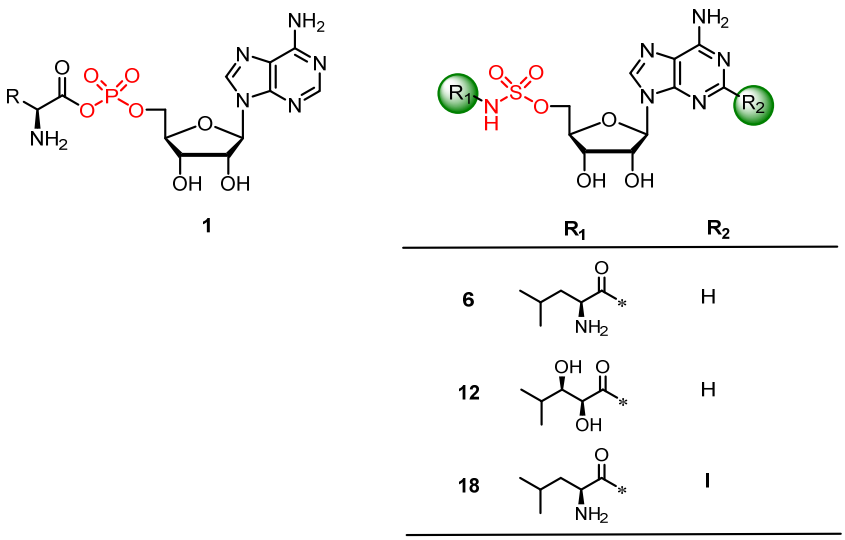


Figure 11. Amionacyl adenylate **1** and leucyladenylate sulfamates.

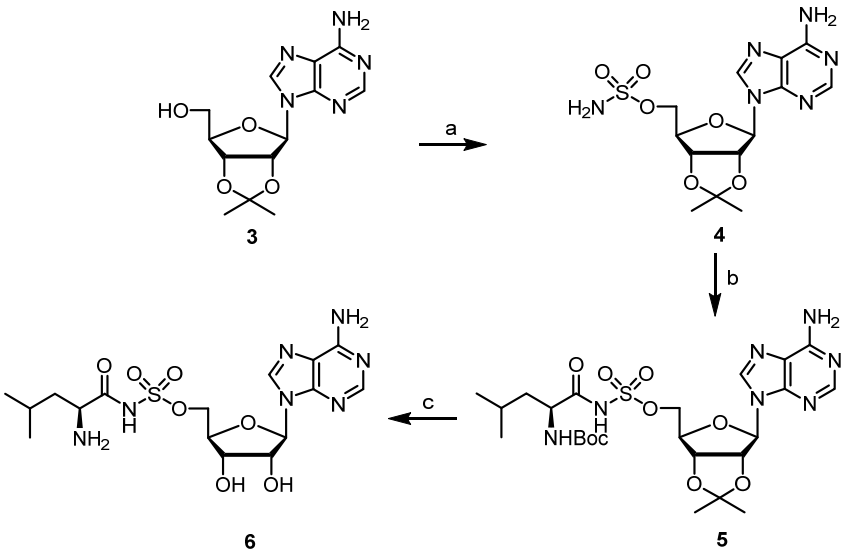
2. Result and Discussion

2.1. Chemistry

Synthesis of leucyladenylate sulfamate **6** began with commercially available 2',3'-isopropylidene adenosine **3** as shown in **Scheme 4**. Compound **3** was reacted with sulfamoyl chloride prepared in a quantitative yield from chlorosulfonyl isocyanate according to previously described procedures.[37] Sulfamoylated adenosine **4** was then coupled with *N*-Boc-leucine to give the leucine adduct **5,** which was subsequently hydrolyzed to leucyladenylate sulfamate **6**.

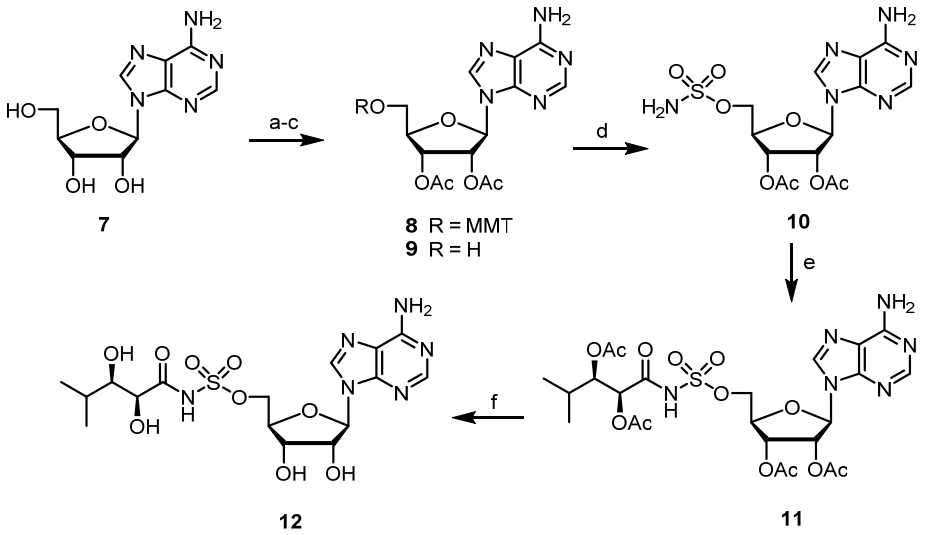
Synthesis of a *syn*-diol analog of leucyladenylate sulfamate **12** was carried out as illustrated in **Scheme 5**. 5'-*O*-Monomethoxytritylation (MMT) of compound **7** followed by acetylation of adenosine provided the fully protected adenosine **8**. Selective removal

of MMT group and subsequent sulfamoylation produced the sulfamate intermediate **10**. (2*S*,3*R*)-dihydroxy-4-methylpentanoic acid (DMPA) protected by *O*-diacetyl was prepared from 4-methyl-2-pentenoic acid in 4 steps by Sharpless asymmetric dihydroxylation, and its configuration was confirmed by following previously described procedures.[36] Amide coupling between compound **10** and the protected chiral acid produced compound **11**, four acetyl groups of which were then removed in the presence of sodium methoxide to yield the final compound **12**.



Scheme 4. Synthesis of the Leucyladenylate Sulfamate **6**.

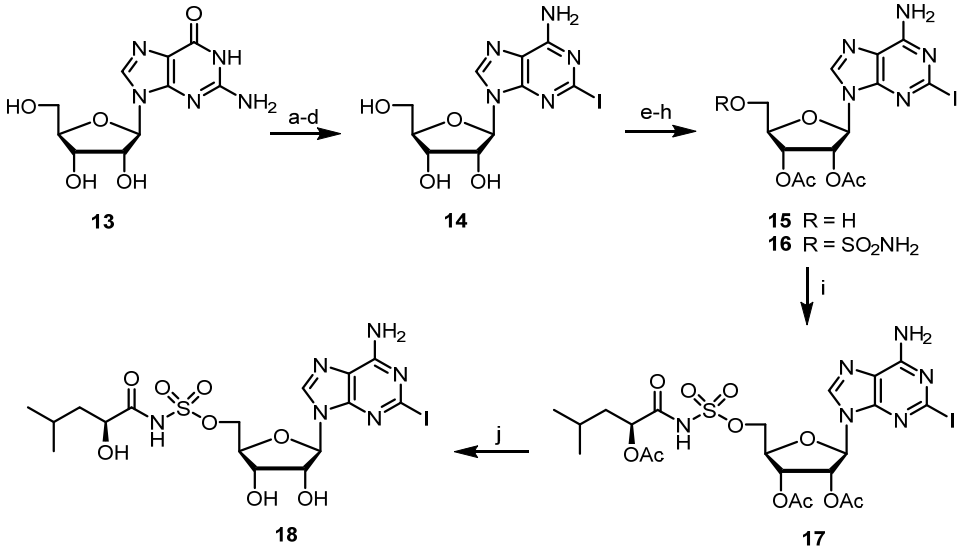
Reagents & conditions: (a) i) NaH, anhyd. THF, 0 ºC, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 ºC to r.t, 5 h; (b) *N*-Boc leucine, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (c) 80% aq. TFA, r.t, 2 h.



Scheme 5. Synthesis of Diol analogue of leucyladenylate sulfamate **12**.

Reagents & conditions: (a) MMTCl, anhyd. pyridine, anhyd. DMF, 0 ºC to r.t, 24 h; (b) acetic anhydride, TEA, DMAP, CH_3CN, 0 ºC to r.t, 4 h; (c) 80% aq. AcOH, r.t, 12 h; (d) i) NaH, anhyd. THF, 0 ºC, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 ºC to r.t, 5 h; (e) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (f) 0.02M NaOMe in MeOH, r.t, 2 h.

Synthesis of a hydroxyl surrogate of leucyladenylate sulfamate **18** is shown in **Scheme 6**. 2-Iodoadenosine (**14**) was synthesized from guanosine (**13**) in 4 steps according to previously described procedures.[37] Compound **14** was converted to the corresponding 5'-*O*-sulfamoylated intermediate **16** by following the route described in **Scheme 5**. (2*S*)-hydroxyisocaproic acid (HICA, *L*-leucic acid) protected by *O*-acetyl was prepared by acetylation of commercially available *L*-leucic acid. A peptide coupling reaction between compound **16** and *O*-acetyl-*L*-leucic acid followed by deprotection of the acetyl group provided the final compound **18**.



Scheme 6. Synthesis of (S)-2-hydroxy-2-iodo-sufamoyl-adenosine **18**.

Reagents & conditions: (a) Ac_2O, TEA, DMAP, CH_3CN, 0 °C to r.t, overmight; (b) $POCl_3$, Et_4NCl, $PhNMe_2$, CH_3CN, r.t to reflux, 1 h; (c) isoamyl nitrite, I_2, CuI, CH_2I_2, THF, reflux, 1 h; (d) 7M NH_3 in MeOH, r.t, overnight; (e-j) the same conditions as (a-f) in scheme 2.

2.2. Biological Activity

Because LRS catalyzes leucylation reaction with its cognate *t*RNA during protein synthesis, we first assessed the inhibitory effects of compounds **6**, **12** and **18** on the catalytic activity of LRS by performing aminoleucylation assays to determine the IC_{50} values. Given that the previously reported acylsulfamate adenylates were found to be potent inhibitors of the corresponding ARSs, these compounds would be likely to exhibit inhibitory effects of LRS. As shown in **Figure 12**, compound **6** proved to be a potent inhibitor of LRS with an IC_{50} value of 22.34 nM. However, compounds **12** and **18** inhibited LRS to a much lesser extent, showing 3- and 15-fold higher IC_{50} values (70.04 nM and 337.1 nM, respectively) than compound **6**. Replacement of the α-amino group of leucine side chain with a hydroxyl group (**12**), and the introduction of a 2-iodo group to

adenine (**18**) weakened the overall binding affinity toward the LRS active site. Moreover, considering fold-changes between compounds, we conclude that modification of the adenine moiety has a higher impact on the catalytic activity than the modification of the leucyl side chain.

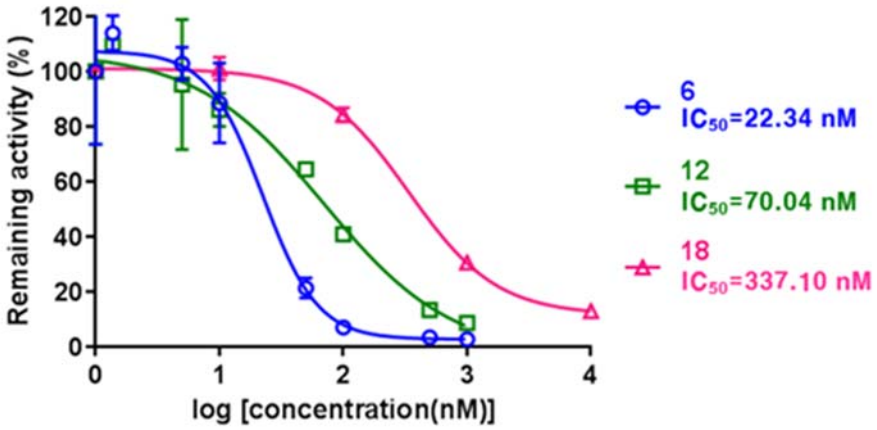


Figure 12. Inhibitory activities of compounds **6**, **12** and **18** for catalytic leucylation.

Next, we determined the effects of compounds **6**, **12**, and **18** on leucine-induced mTORC1 activation using the immunoblotting method. In our previous study, pretreatment of leucinol analogs blocked leucine-induced phosphorylation of S6K, an mTORC1 substrate, by directly interacting with LRS[24]; because the newly designed compounds have additional adenosine moieties, we would expect to observe stronger inhibitory effects for S6K phosphorylation. We pretreated HEK293 cells with each compound at different concentrations as well as with rapamycin as a control at 100 nM, and then activated mTORC1 by treating the cells with leucine for 10 min. As shown in

Figure 13, pretreatment of rapamycin blocked phosphorylation of S6K, whereas pretreatment of compound **6** did not affect S6K phosphorylation at all (**Figure 13A**). Notably, compounds **12** and **18** showed a dose-dependent inhibition of mTORC1 in HEK293 cells (**Figure 13B, 13C**) while compound **18** appeared to be more potent than compound **12**. Interestingly, these three sulfamates inhibited leucine-induced mTORC1 activation in the order of **18** > **12** > **6**, in contrast to the order of their inhibitory effects for the catalytic reaction (**6** > **12** > **18**). This apparently opposite trend suggests that LRS-mediated mTORC1 activation is independent of the leucylation activity of LRS on its cognate tRNA, which is again in agreement with previously reported observations.[13, 24] More interestingly, the side chain modifications of an α-hydroxyl group in the leucyl side chain and a 2-iodo group in the adenine, both of which adversely affected the catalytic activity of LRS, resulted in favorable effects for the mTORC1 inhibition.

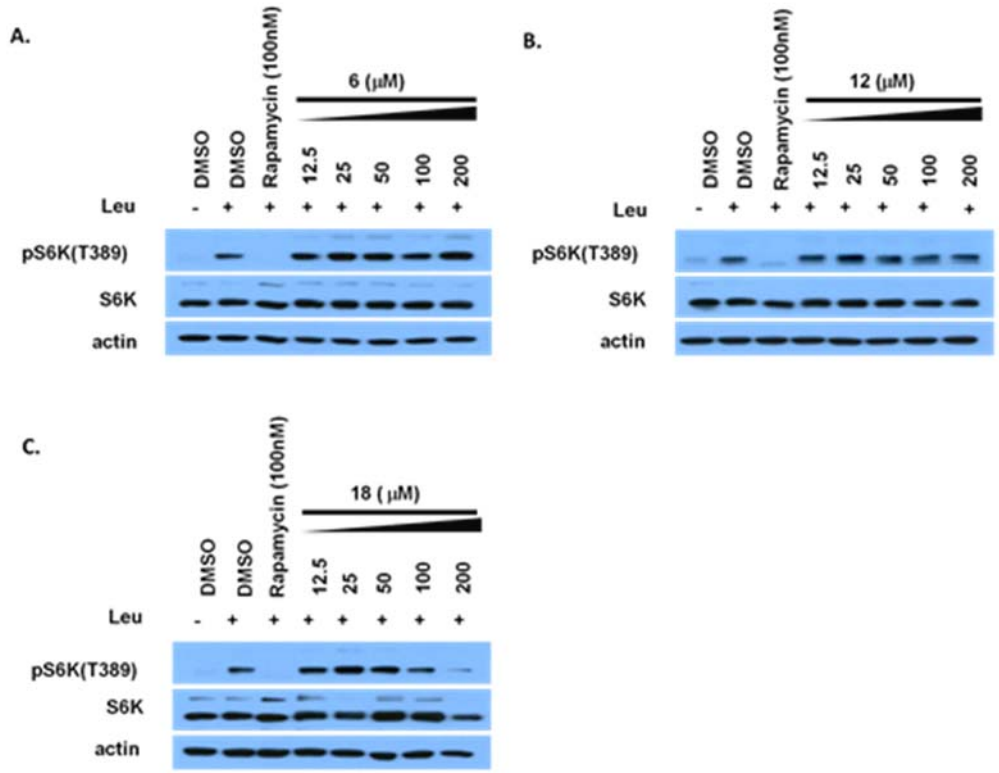


Figure 13. Dose-dependent inhibition of leucine-induced mTORC1 activation of c ompounds **6**, **12** and **18** in HEK293 cells

To further investigate the mechanisms of these sulfamates in LRS-mediated mTORC1 activation, we performed *in vitro* mTORC1 kinase assays using purified mTOR. As demonstrated in **Figure 14**, compounds **12** and **18** both did not inhibit the kinase activity of the purified mTOR at 200 μM, suggesting that these compounds are highly selective toward LRS, and did not act as an ATP-competitive inhibitor of the mTOR complex. We believe that these two sulfamates, **12** and **18**, inhibited the mTORC1 activity by selectively blocking the LRS-mediated mTORC1 activation pathway, rather than directly interacting with mTOR.

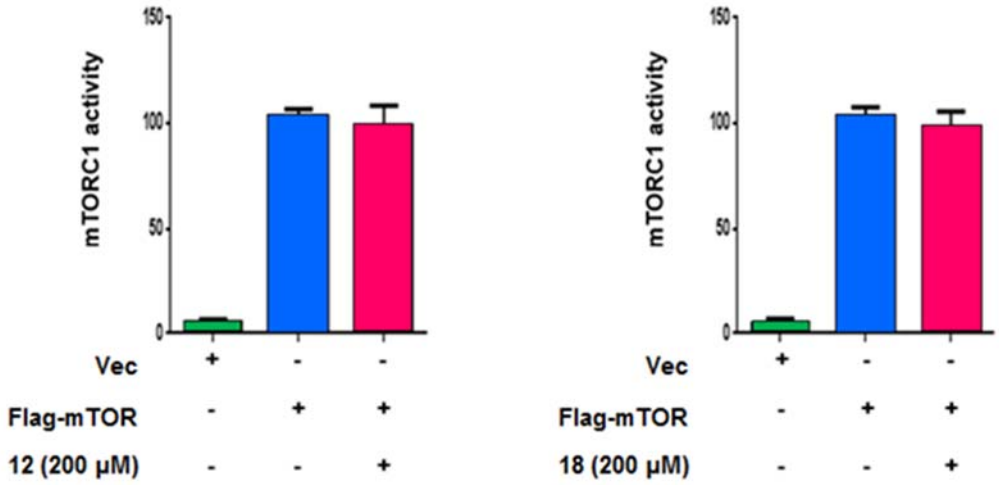


Figure 14. Effects of compounds **12** and **18** on the kinase activity of the purifie d mTOR.

Finally, to examine the anti-cancer activity of leucyladenylate sulfamates, we performed the sulforhodamine B (SRB) colorimetric assays for cytotoxicity.[38] We treated compounds **6**, **12**, and **18** with six different types of cancer cell lines together with etoposide as a positive control. To determine cancer cell selectivity, we also treated all four compounds with normal human lung epithelial cells (MRC-5), with the measured IC_{50} values shown in **Table 1**. Compound **6**, the most potent LRS inhibitor, exhibited the greatest cytotoxicity that was even greater than that of etoposide in all types of cells, except A549 and SNU638. Given that LRS plays a crucial role in protein synthesis, it is not surprising that compound **6** is highly cytotoxic in both cancer cells and normal cells. In contrast, compounds **12** and **18** showed selective cytotoxicity against cancer cells. Specifically, compound **18** showed potent cytotoxicity against colon cancer cells (HCT116) and leukemia cells (K562) while exhibiting much less cytotoxicity against normal cells compared with compound **6** and etoposide. This result is particularly

promising because several recent studies have reported that hyperactive mTORC1 is one of the distinctive features in human colorectal cancer.[39,40] suggesting that compound **18** exerted colon cancer specific cytotoxicity by selective inhibition of mTORC1.

Table 1. Relative cell growth inhibition of compounds **6**, **12** and **18** for various cancer cell types and normal cells.[a]

IC_{50} (μM)	A549	HCT116	K562	MDA-MB-231	SK-HEP-1	SNU638	MRC5
6	0.59	0.24	0.4	0.73	0.54	0.84	5.9
12	1.64	1.44	2.22	7.01	6.81	8.98	>50
18	1.75	0.54	1.06	12.6	5.63	5.7	>50
Etoposide	0.24	1.25	1.79	7.35	0.25	0.56	12.7

[a]A549: lung cancer cells, MDA-MB-231: breast cancer cells, SK-Hep-1: liver cancer cells, SNU638: stomach cancer cells, HCT116: colon cancer cells, K562: leukemia cells, MRC5: lung normal epithelial cell

3. Conclusion

We have developed leucyladenylate sulfamate derivatives that directly interact with LRS to inhibit the mTORC1 pathway. Compound **6** inhibited the catalytic activity of LRS but did not affect the leucine-induced mTORC1 activation, whereas compound **18** inhibited mTORC1 activation, while it also inhibited the catalytic activity of LRS to a much lesser degree compared to compounds **6** and **12**. Furthermore, both compounds **12** and **18** did not affect the kinase activity of the purified mTOR, indicating that the mTORC1-specific activity of these compounds arose from blocking the leucine-sensing ability of LRS rather than from interacting with mTOR directly. Cytotoxicity screening in various types of cancer cells and normal cells revealed that compound **6** showed the greatest cytotoxicity, probably due to a non-specific inhibition of LRS. Compounds **12** and **18** demonstrated

cytotoxicity against all types of cancer cells but not against normal cells. Most notably, compound **18** exerted highly specific cytotoxicity against colon cancer cells that are known to have hyperactive mTORC1. We believe that compound **18** may serve as a useful tool to study the role of LRS in the mTORC1 pathway but may also offer a novel treatment option for human colorectal cancer.

4. Experimental

4.1. General Experimental

All chemical reagents were commercially available. Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230-400 mesh, Merck. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded on JEOL JNM-LA 300 [300 MHz (^{1}H), 75 MHz (^{13}C)] and Bruker Avance 400 MHz FT-NMR [400 MHz (^{1}H), 100 MHz (^{13}C)] spectrometers. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC-MS and 6460 Triple Quad LC/MS. All final compounds were purified to >95% purity, as determined by high-performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1120 Compact LC (G4288A) instrument using an Agilent Eclipse Plus C18 column (4.6 x 250 mm, 5 μm) and a Daicel Chiralcel OD-H column (4.6 x 250 mm, 5 μm).

4.2. Procedures and Chemical spectra

4.2.1. ((3a*R*,4*R*,6*R*,6a*R*)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetra-hydrofuro[3,4-d][1,3]dioxol-4-yl)methyl sulfamate (4).

Compound **4** was prepared by following the reported procedure.[32] Yield 69%, white solid; ^{1}H-NMR (300MHz, CD_3OD) δ 8.27 (s, 1H, CH), 8.22 (s, 1H, CH), 6.24 (d, 1H, CH, *J* = 2.55 Hz), 5.42 (dd, 1H, CH, *J* = 6.24 Hz, 2.58 Hz), 5.13 (dd, 1H, CH, *J* = 6.21 Hz, 2.73 Hz), 4.51 (m, 1H, CH), 4.32 (dd, 1H, CH, *J* = 10.62 Hz, 4.59 Hz), 4.24 (dd, 1H, CH, *J* = 10.62 Hz, 5.31 Hz), 1.60 (s, 3H, CH_3), 1.39 (s, 3H, CH_3)

4.2.2. ((3a*R*,4*R*,6*R*,6a*R*)-6-(6-amino-9H-purin-9-yl)-2,2-dimethyltetra hydrofuro[3,4-d][1,3]dioxol-4-yl)methyl ((tert-butoxy carbonyl)-*L*-leucyl)sulfamate (5).

To a solution of compound **4** (0.54 mmol) in anhydrous MC (25 mL) was added *N*-Boc leucine (0.81 mmol) and DMAP (0.01 mmol) at 0ºC. 1M DCC in MC (0.81 mmol) was added dropwise, stirred for 2 h at room temperature. The solution was filtered on celite pad, washed with EtOAc (20 mL), and then concentrated. The filtrate was purified by column chromatography over silica gel (EtOAc:MeOH=10:1) to give the compound **5** (0.46 mmol). Yield 85%, white solid; ^{1}H-NMR (300MHz, CD_3OD) δ 8.47 (s, 1H, CH), 8.21 (s, 1H, CH), 6.22 (d, 1H, CH, *J* = 3.30 Hz), 5.33 (dd, 1H, CH, *J* = 5.88 Hz, 3.48 Hz), 5.10 (d, 1H, CH, *J* = 5.67 Hz), 4.53 (m, 1H, CH), 4.23 (d, 2H, CH_2, *J* = 3.48 Hz), 4.06 (m, 1H, CH), 1.69 (m, 1H, CH), 1.60 (s, 3H, CH_3), 1.52 (m, 2H, CH_2), 1.43 (s, 9H, CH_3), 1.40 (s, 3H, CH_3), 0.93 (dd, 6H, CH_3, *J* = 6.60 Hz, 2.76 Hz)

4.2.3. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetra hydrofuran-2-yl)methyl (*L*-leucyl) sulfamate trifluoroacetate salt (6).

The compound **5** (0.38 mmol) was dissolved in 80% aquous TFA (2mL) and stirred for 2 h at room temperature. The reaction mixture was evaporated and washed with EtOAc (20 mL). Aqueous layer was concentrated under reduced pressure to give crude pale yellow solid. The solid was purified by ion-exchange resin (HP20SS) to give the compound **6** (0.16 mmol). Yield 42%, white solid; ^{1}H-NMR (500MHz, CD_3OD) δ 8.49 (s, 1H, CH), 8.20 (s, 1H, CH), 6.07 (d, 1H, CH, *J* = 5.30 Hz), 4.62 (t, 1H, CH, *J* = 5.10 Hz), 4.39-4.29 (m, 4H, , 2 CH, CH_2), 3.61 (dd, 1H, CH, *J* = 8.50 Hz, 4.85 Hz), 1.78 (m, 2H, CH_2), 1.57 (m, 1H, CH), 0.95 (dd, 6H, CH_3, *J* = 15.95 Hz, 6.00 Hz); HRMS-FAB *m/z* $[M+H]^+$ $C_{16}H_{25}N_7O_7SH^+$ calcd 460.1536, Found: 460.4898.

4.2.4. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-9H-purin-9-yl)-5-(((4-methoxyphenyl) diphenylmethoxy)methyl)tetrahydrofuran-3,4-diyl diacetate (8).

5'-O-Monomethoxytrityl (MMT) protected adenosine (2.27 mmol) was prepared by following the reported procedure.[38] To a solution of 5'-O-MMT protected adenosine (2.05 mmol) in acetonitrile (50 mL) at 0ºC was added DMAP (0.21 mmol), TEA (6.15 mmol) and acetic anhydride (6.15 mmol). The mixture was stirred for 4hr at room temperature. Aqueous portion was extracted with EtOAc (50 mL). The organic phase was dried over $MgSO_4$ and concentrated, which was purified by column chromatography (EtOAc:MeOH = 40:1, v/v) to give compound **8** (1.6 mmol). Yield 78%, white solid; ^{1}H-NMR (300MHz, $CDCl_3$) δ 8.01 (S, 1H, CH), 7.90 (S, 1H, CH), 7.42 (m, 4H, Ar), 7.32 (m, 4H, Ar), 7.25 (m, 4H, Ar), 6.18 (m, 4H, Ar), 6.26 (d, 1H, CH, *J* = 6.6 Hz), 6.07 (t, 1H, CH, *J* = 5.3

Hz), 5.67 (dd, 1H, CH, *J* = 5.1 Hz, 3.1 Hz), 4.31 (m, 1H, CH), 3.78 (S, 3H, CH_3), 3.46 (m, 1H, CH), 2.10 (S, 3H, CH_3), 2.05 (s, 3H, CH_3)

4.2.5. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-9H-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diyl diacetate (9).

80% aquous AcOH (100 mL) was slowly added to compound **8** (1.52 mmol) and the reaction mixture was stirred for 12 h at room temperature. The mixture was evaporated, neutralized with $NaHCO_3$ and extrated with EtOAc (150 mL x 2). Organic layer was combined, dried over $MgSO_4$ and evaporated. The residue was purified by column chromatography (EtOAc:MeOH = 20:1, v/v) to give compound **9** (0.97 mmol). Yield 64%, white solid; ^{1}H-NMR (300MHz, $CDCl_3$) δ 8.32 (s, 1H, CH), 7.85 (S, 1H, CH), 6.26 (S, 2H, NH_2), 6.03 (d, 2H, CH_2, *J* = 2.0 Hz), 5.70 (m, 1H, CH), 4.37 (s, 1H, CH), 3.99 (dd, 1H, CH, *J*= 13.0 Hz, 1.4 Hz), 3.86 (d, 1H, CH, *J* = 12.3 Hz), 2.18 (s, 3H, CH_3), 2.02 (s, 3H, CH_3)

4.2.6. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-9H-purin-9-yl)-5-((sulfamoyloxy) methyl)tetrahydrofuran-3,4-diyl diacetate (10).

Compound **10** was prepared by following the procedure described for compound **4**. Yield 93%, white solid; ^{1}H-NMR (300MHz, $CDCl_3$) δ 8.34 (s, 1H, CH), 8.00 (s, 1H, CH), 6.16 (d, 1H, CH, *J* = 6.1 Hz), 5.91 (t, 1H, CH, *J* = 5.9 Hz), 5.72 (m, 1H, CH), 2.16 (s, 3H, CH_3), 2.07 (s, 3H, CH_3)

4.2.7. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-9H-purin-9-yl)-5-(((*N*-((2*S*,3*R*)-2,3-diacetoxy-4-methylpentanoyl)sulfamoyl)oxy)methyl)tetrahydrofuran-3,4-diyl diacetate (11).

Compound **11** was prepared by following the procedure described for compound **5**. Yield 79%, white solid; ^{1}H-NMR (300MHz, CD_3OD) δ 8.47 (s, 1H, CH), 8.34 (s, 1H, CH), 6.29 (d, 1H, CH, *J* = 5.3 Hz), 5.85 (t, 1H, CH, *J* = 5.49 Hz), 5.68 (dd, 1H, CH, *J* = 5.67 Hz, 4.0 Hz), 5.14 (dd, 1H, CH, *J* = 8.8 Hz, 2.7 Hz), 5.07 (d, 1H, CH, *J* = 2.7 Hz), 4.52 (m, 2H, CH, NH), 3.68 (s, 1H, CH), 2.14 (s, 3H, CH_3), 2.03 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.04 (m, 1H, CH), 0.94 (d, 3H, CH_3, *J* = 2.5 Hz), 0.91 (d, 3H, CH_3, *J* = 2.4 Hz)

4.2.8. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetra hydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoyl) sulfamate (12).

Compound **11** (0.056 mmol) was dissolved in 0.02 M sodium methoxide solution in methanol (3 mL) and stirred for 2 h at room temperature. DOWEX 50WX8 hydrogen form resin (10 mg) was added in portions, filtered and concentrated to afford compound **12** (0.03 mmol). Yield 56%, white solid; ^{1}H-NMR (600MHz, CD_3OD) δ 8.52 (s, 1H, CH), 8.18 (s, 1H, CH), 6.08 (d, 1H, CH, *J* = 2.8 Hz), 4.64 (t, 1H, CH, *J* = 2.5 Hz), 4.40 (m, 1H, CH), 4.31 (m, 3H, CH), 4.05 (d, 1H, CH, *J* = 1.0 Hz), 3.51 (dd, 1H, CH, *J* = 4.4 Hz, 1.0 Hz), 1.86 (m, 1H, CH), 1.01 (d, 3H, CH_3, *J* = 3.2 Hz), 0.94 (d, 3H, CH_3, *J* = 3.2 Hz); HRMS-ESI *m/z* $[M+H]^+$ $C_{16}H_{24}N_6O_9SH^+$ calcd 477.1325, Found: 477.1395.

4.2.9. (2*R*,3*R*,4*S*,5*R*)-2-(6-amino-2-iodo-9H-purin-9-yl)-5-(hydroxyl methyl)tetrahydrofuran-3,4-diol (14).

Compound **14** was prepared by following the reported procedure.[34] Yield 11% in 4 steps, yellow solid; ^{1}H-NMR (300MHz, CD_3OD) δ 8.20 (s, 1H, CH), 5.89 (d, 1H, CH, *J* = 6.03 Hz), 4.66 (t, 1H, CH, *J* = 5.31 Hz), 4.30 (dd, 1H, CH, *J* = 5.10 Hz, 3.09 Hz), 4.15-4.12 (m, 1H, CH), 3.88 (dd, 1H, CH, *J* = 12.45 Hz, 2.73 Hz), 3.74 (dd, 1H, CH, *J* = 12.45 Hz, 2.94 Hz), 2.18 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 2.11 (s, 3H, CH_3)

4.2.10. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-2-iodo-9H-purin-9-yl)-5-(hydroxy methyl)tetrahydrofuran-3,4-diyl diacetate (15).

Compound **15** was prepared by following the procedure described for compounds **8** and **9**. Yield 67% in 3 steps, colorless oil; ^{1}H-NMR (300MHz, $CDCl_3$) δ 7.77 (s, 1H, CH), 6.47 (br, 1H, OH), 5.97 (d, 1H, CH, *J* = 7.68 Hz), 5.91 (m, 1H, CH), 5.67 (dd, 1H, CH, *J* = 5.13 Hz, 1.29 Hz), 4.36 (d, 1H, CH, *J* = 1.08 Hz), 4.51 (dd, 1H, CH, *J* = 13.02 Hz, 1.47 Hz), 3.88 (d, 1H, CH, *J* = 11.73 Hz), 2.17 (s, 3H, CH_3), 2.11 (s, 3H, CH_3)

4.2.11. (2*R*,3*R*,4*R*,5*R*)-2-(6-amino-2-iodo-9H-purin-9-yl)-5-((sulfamoyl oxy)methyl)tetrahydrofuran-3,4-diyl diacetate (16).

Compound **16** was prepared by following the procedure described for compound **10**. Yield 86%, white solid; ^{1}H-NMR (300MHz, $CDCl_3$) δ 7.93 (s, 1H, CH), 6.14 (d, 1H, CH, *J* = 5.49 Hz), 5.84 (br, 2H, NH_2), 5.78 (t, 1H, CH, *J* = 5.31 Hz), 5.71 (t, 1H, CH, *J* = 4.02 Hz), 4.53 (d, 2H, CH_2, *J* = 3.48 Hz), 4.47 (m, 1H, CH), 2.16 (s, 3H, CH_3), 2.09 (s, 3H, CH_3)

4.2.12. (2*R*,3*R*,4*R*,5*R*)-2-(((*N*-((*S*)-2-acetoxy-4-methylpentanoyl)sulfamoyl)oxy)methyl)-5-(6-amino-2-iodo-9H-purin-9-yl)tetrahydrofuran-3,4-diyl diacetate (17).

Compound **17** (0.03 mmol) was prepared by following the procedure described for compound **11**. Yield 81%, white solid; ^{1}H-NMR (300MHz, $CDCl_3$) δ 8.31 (s, 1H, CH), 6.28 (d, 1H, CH, *J* = 6.69 Hz), 5.75 (m, 1H, CH), 5.54 (m, 1H, CH), 4.70 (m, 1H, CH), 4.42-4.34 (m, 2H, CH_2), 2.14 (s, 3H, CH_3), 1.98 (s, 6H, CH_3), 1.64 (m, 2H, CH_2), 1.50 (m, 1H. CH), 0.79(dd, 6H, CH_3, *J* = 13.17 Hz, 6.60 Hz)

4.2.13. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-iodo-9H-purin-9-yl)-3,4-dihydroxy tetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl) sulfamate (18).

Compound **18** was prepared by following the procedure described for compound **12**. Yield 67%, white solid; ^{1}H-NMR (500MHz, CD_3OD) δ 8.26 (s, 1H, CH), 5.98 (d, 1H, CH, *J* = 4.90 Hz), 4.55 (t, 1H, CH, *J* = 4.85 Hz), 4.50 (dd, 1H, CH, *J* = 11.20 Hz, 2.80 Hz), 4.44 (dd, 1H, CH, *J* = 11.2 Hz, 3.40 Hz), 4.36 (t, 1H, CH, *J* = 4.70 Hz), 4.28 (q, 1H, CH, *J* = 3.60 Hz), 4.03 (t, 1H, CH, *J* = 6.30 Hz), 1.83-1.79 (m, 1H, CH), 1.52-1.47 (m, 2H, CH_2), 0.90 (dd, 6H, CH_3, *J* = 6.60 Hz, 2.25 Hz); HRMS-ESI *m/z* $[M+H]^+$ $C_{16}H_{23}IN_6O_8SH^+$ calcd 587.0343, Found 587.0410.

IV. Part 2-2. Structure Activity Relationship (SAR) Studies of Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

1. Design background & strategy

Previously, we designed a new series of compounds based on the structure of aminoacyl adenylate which is well known enzymatic reaction intermediate of ARSs and reported the three representative compounds among the library of leucyladenylate sulfamates. Here in, we describe the synthesis and inhibitory activities against mTORC1 of other leucyladenylate sulfamtes and structure-activity relationship of leucyladenylate sulfamte derivatives. We modified adenine, ribose ring and leucyl side chains moieties and evaluated their biological activity. In R^1 group, we introduced N^6-methylamino group and oxygen instead of N^6-amino group of adenine and 2-cholro, 2-iodo and alkyl chain for R^2 group modification. Also, we designed arabinose, 2'-deoxy ribose, 3'-deoxy ribose, 3'-deoxy-3'amino ribose and bicyclic structure of 3'-deoxy-3'-amino ribose for the ribose ring part. Furthermore, for the leucyl side chain modification, we substituted (*S*)-amino group in α-position of leucine with (*S*)-hydroxyl, (*R*)-hydroxyl, (*S*)-chloro, (*S*)-thio hydroxyl groups etc. and synthesized the leucyladenylate sulfamte derivatives and evaluated suppression of mTORC1 activation.

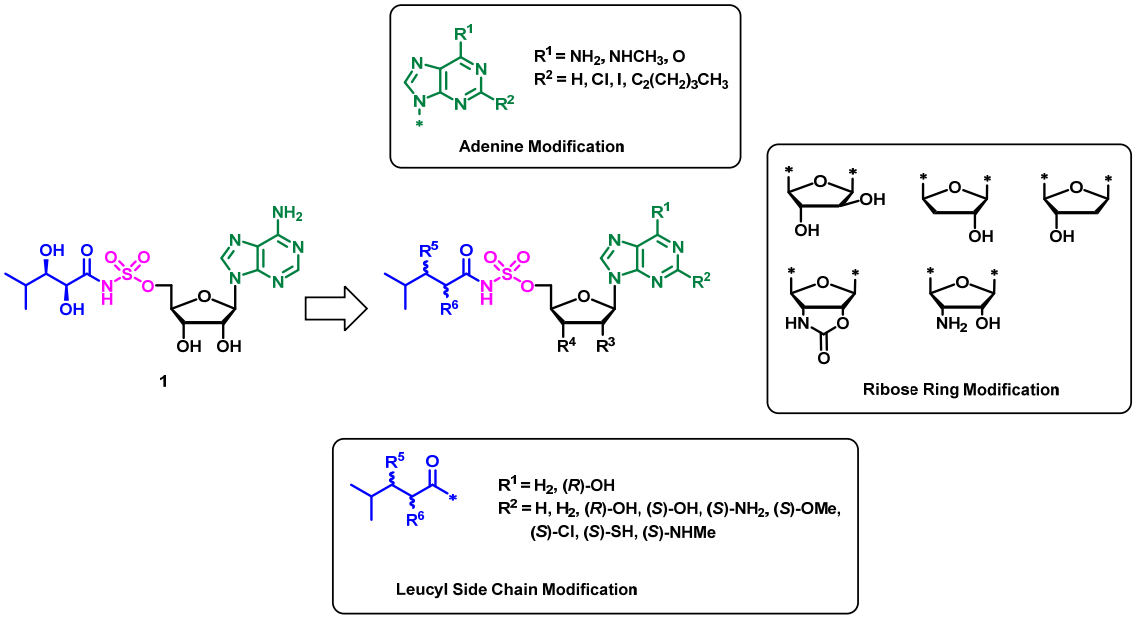
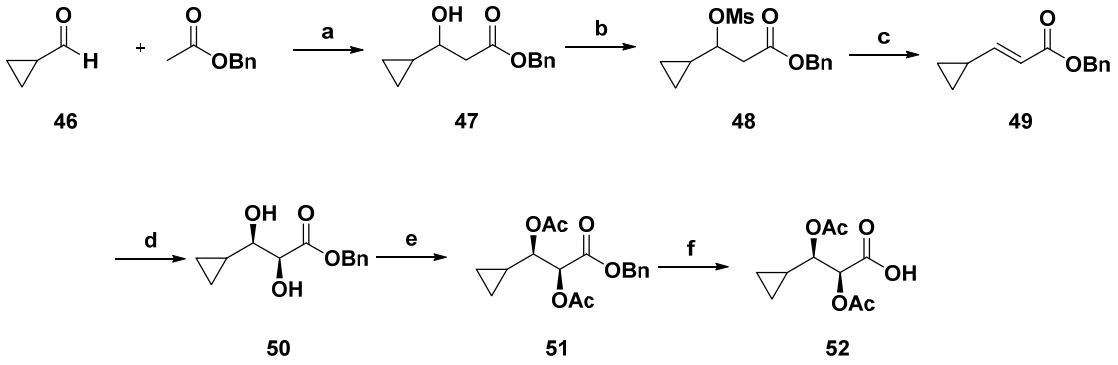


Figure 15. (2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoylsulfamoyl adenylate (**1**) and its derivatives for SAR study.

2. Result and Discussion

2.1. Chemistry

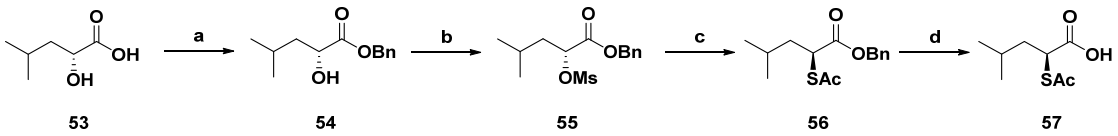
Synthesis of (2*S*,3*R*)-2,3-diacetoxy-3-cyclopropylpropanoic acid (**52**) began with commercially available cyclopropane carboxaldehyde (**46**) as illustrated in **scheme 7**. Aldol reaction of aldehyde **46** with benzyl acetate provided β-hydroxy ketone **47**. After introducing mesyl leaving group, condensation reaction under basic condition provided α, β-unsaturated ketone **49**. Sharpless asymmetric dihydroxylation followed by acetylation and debenzylation produced compound **52**.



Scheme 7. Synthesis of (2*S*,3*R*)-2,3-diacetoxy-3-cyclopropylpropanoic acid **(52)**.

Reagents & conditions: (a) LHMDS, anhyd.THF, -78 ^{o}C, overnight, 52%; (b) MsCl, TEA, MC, 0 ^{o}C to r.t, overnight, 56%; (c) DBU, DMF, 0 ^{o}C to r.t, 2 h, 99%; (d) AD Mix β, H_2O:*t*-BuOH = 1:1, r.t, 12 h, 79%; (e) Ac_2O, TEA, DMAP, ACN, r.t, 12 h, 96%; (f) Pd/C, H_2, MeOH, r.t, 2 h, 99%.

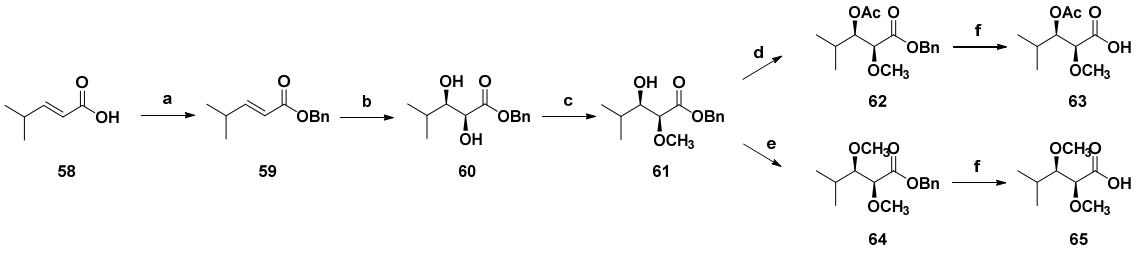
(*S*)-2-(acetylthio)-4-methylpentanoic acid (**57**) was prepared from commercailly available D-α-Hydroxy isocaproic acid (**53**) in 4 steps as described in **scheme 8**. After benzyl protection of carboxylic acid, mesylate group was converted to thioacetate group with inversed steroechemistry followed by removal of benzyl group afforded compound **57**.



Scheme 8. Synthesis of (*S*)-2-(acetylthio)-4-methylpentanoic acid **(57)**.

Reagents & conditions: (a) (i) Cs_2CO_3, MeOH/H_2O (5:1), r,t, 30 min (ii) BnBr, DMF, 0 ^{o}C to r.t, 12 h, 99%; (b) MsCl, TEA, MC, 0 ^{o}C to r.t, 2 h, 99%; (c) KSAc, ACT, reflux, 5 h, 99%; (d) BCl_3, CH_2Cl_2, -78 ^{o}C to 0 ^{o}C, 1 h, 40%.

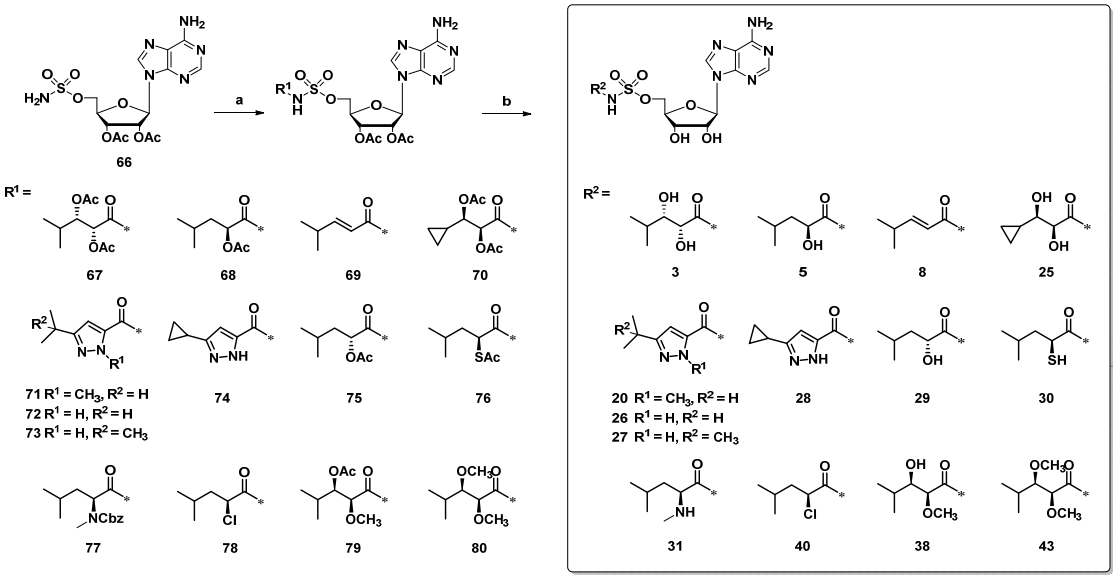
Compounds **63** and **65** were prepared from commercially available 4-methyl-2-pentenoic acid (**58**) in 5 steps as shown in **scheme 9**. Sharpless asymmetric dihydroxylation provided compound **60**, monomethylation of α-hydroxyl group provided compound **61**, then acetylation and deprotection of the benzyl group produced (2*S*,3*R*)-3-acetoxy-2-methoxy -4-methylpentanoic acid (**63**). Methylation of β-hydroxyl group of compound **60** produced dimethoxy product **64** followed by deprotection of the benzyl group afforded (2*S*,3*R*)-2,3-dimethoxy-4-methylpentanoic acid (**65**).



Scheme 9. Synthesis of (2*S*,3*R*)-3-acetoxy-2-methoxy -4-methylpentanoic acid (**63**) and (2*S*,3*R*)-2,3-dimethoxy-4-methylpentanoic acid (**65**).

Reagents & conditions: (a) K_2CO_3, BnBr, ACT, reflux, overnight, 99%; (b) AD mix β, H2O:*t*-BuOH, r.t, overnight, 87%; (c) Ag_2O, MeI, CH_2Cl_2, refux, 36 h, 20%; (d) Ag_2O, MeI, CH_2Cl_2, refux, 36 h, 69%;(f) Pd/C, H_2, Methanol, 99%.

Synthesis of leucyladenylate sulfamate derivatives **3**, **5**, **8**, **20**, **25-31**, **38**, **40**, **43** were carried out as illustrated in **Scheme 10**. Sulfamate intermediate **66** was prepared following a previous report.[32] Amide coupling between compound **66** and the protected chiral acids and pyrazole-5-carboxylic acid derivatives produced intermediates **67-80**, protection groups of each compounds were then removed to produce the corresponding final compounds.

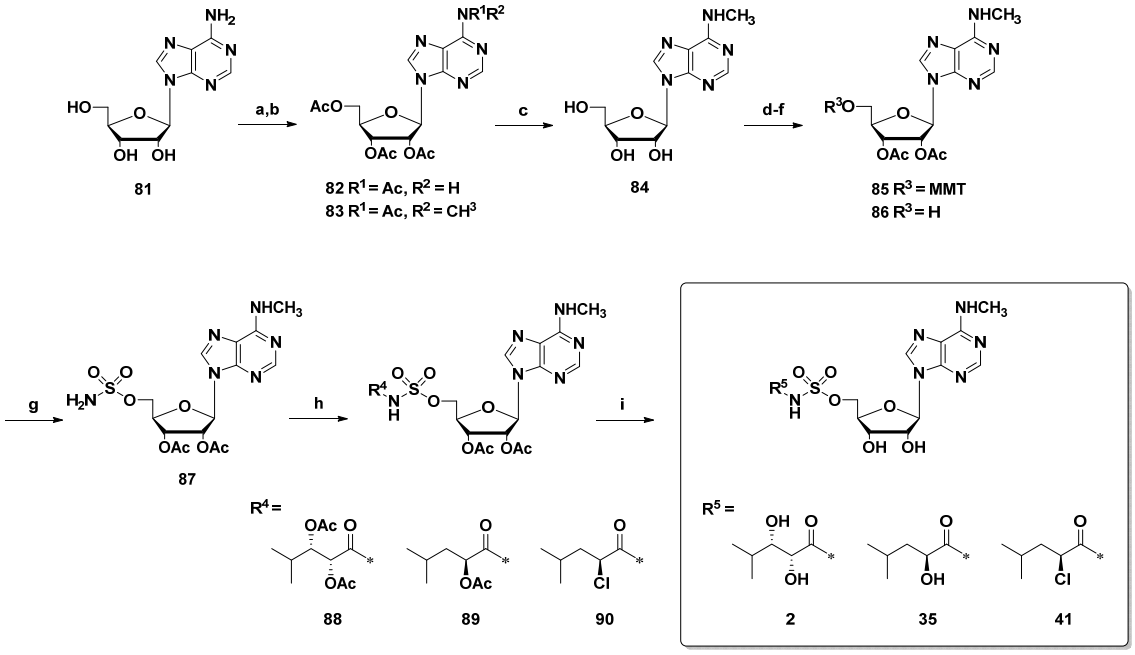


Scheme 10. Synthesis of aminoacyladenylate analogues.

Reagents & conditions: (a) corresponding acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h, 45~80%; (b) 0.02 M NaOMe in MeOH, r.t, 2 h, 68~85%.

Synthesis of N^6-methyladenylate analogues is illustrated in **scheme 11**. N^6-methyladenosine (**84**) was synthesized from adenosine (**81**) in 3 steps, then corresponding 5'-*O*-sulfamoylated intermediate **87** was prepared by same route described in **scheme 5**. (2*R*,3*S*)-dihydroxy-4-methylpentanoic acid (DMPA) protected by *O*-diacetyl was prepared from commercially available 4-methyl-2-pentenoic acid in 4 steps by Sharpless asymmetric dihydroxylation, and its configuration was confirmed by following previously described procedures.[38] (2*S*)-hydroxyisocaproic acid (HICA, *L*-leucic acid) protected by *O*-acetyl was prepared by acetylation of commercially available *L*-leucic acid. Amide coupling between compound **87** and the corresponding protected chiral acids produced compounds **88** and **89**, respectively. Acetyl groups of which were then removed in the presence of sodium methoxide to yield the final compounds **2** and **35**. A peptide

coupling reaction between compound **87** and commercially available (*S*)-2-chloro-4-methylpentanoic acid followed by deprotection of the acetyl groups provided the final compound **41**.

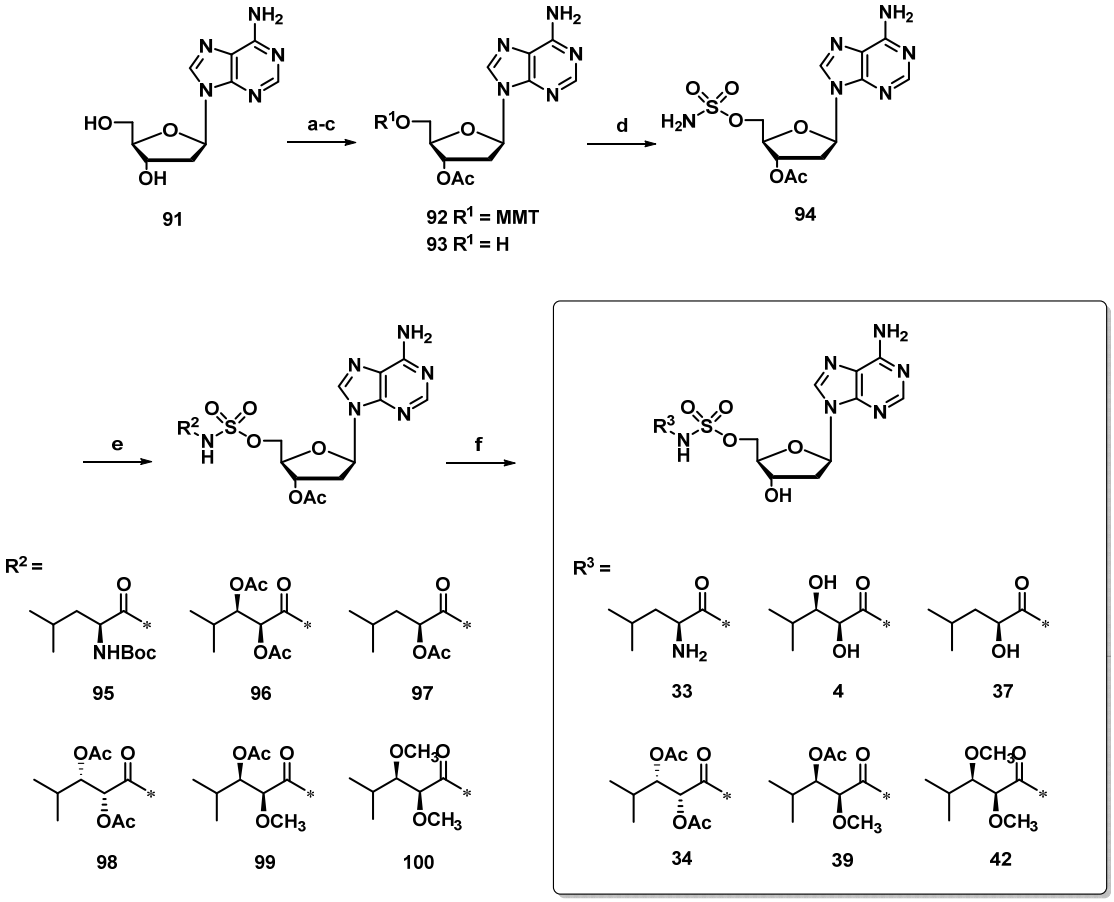


Scheme 11. Synthesis of N^6-methyladenylate analogues.

Reagents & conditions: (a) Ac_2O, pyidine, 60 ^oC, 8 h; (b) CH_3Br, DBU, MeCN, r.t. (c) 7M NH_3 in MeOH (d) MMTCl, anhyd. pyridine, anhyd. DMF, 0 ^oC to r.t, 24 h; (e) Ac_2O, TEA, DMAP, CH_3CN, 0 ^oC to r.t, 4 h; (f) 80% aq. AcOH, r.t, 12 h; (g) i) NaH, anhyd. THF, 0 ^oC, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 ^oC to r.t, 5 h; (h) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (i) 0.02 M NaOMe in MeOH, r.t, 2 h.

Synthesis of 2'-deoxyadenylate analogues is shown in **scheme 12**. 5'-*O*-sulfamoylated intermediate **94** was prepared in 4 steps following the same route described in **scheme 5**. DCC coupling with corresponding protected chiral acids provided intermediates **95-100**,

then removal of protection groups yielded each final compounds.

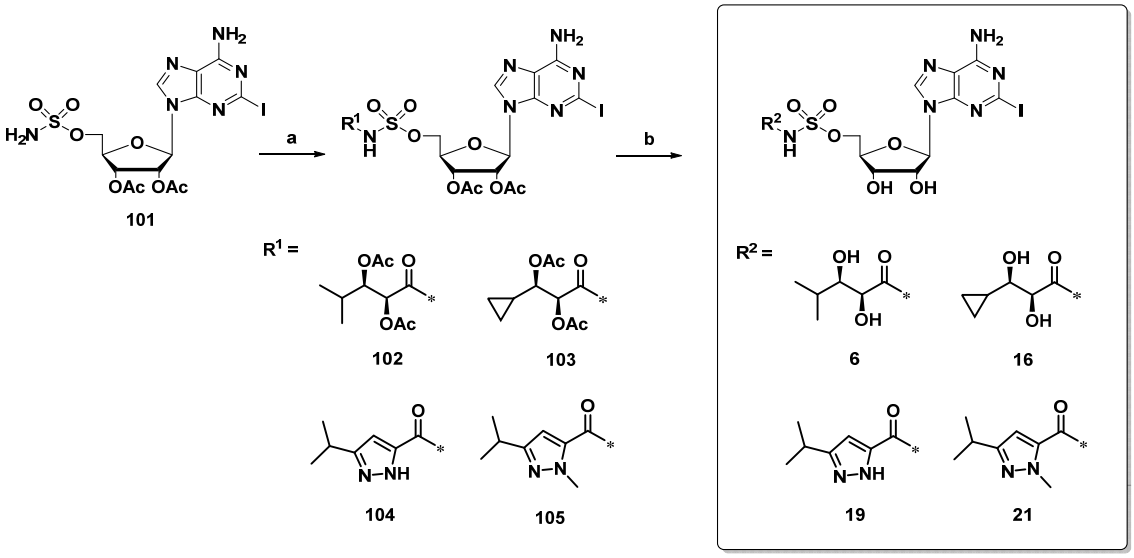


Scheme 12. Synthesis of 2'-deoxyadenylate analogues.

Reagents & conditions: (a) MMTCl, anhyd. pyridine, anhyd. DMF, 0 °C to r.t, 24 h, 53%; (b) Ac2O, TEA, DMAP, CH_3CN, 0 °C to r.t, 4 h, 89%; (c) 80% aq. AcOH, r.t, 12 h, 48%; (d) i) NaH, anhyd. THF, 0 °C, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 °C to r.t, 5 h, 77%; (e) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h, 25~45%; (f) 0.02M NaOMe in MeOH, r.t, 2 h, 30~50%.

5'-*O*-sulfamoylated 2-iodoadenosine (**101**) was synthesized from guanosine in 4 steps by following the route described in **Scheme 6**. Peptide coupling between compound **101** and the protected chiral acids and pyrazole-5-carboxylic acid derivatives produced

intermediates **102-105** followed by deprotection of four acetyl groups produced corresponding final compounds **6**, **16**, **19** and **21**.

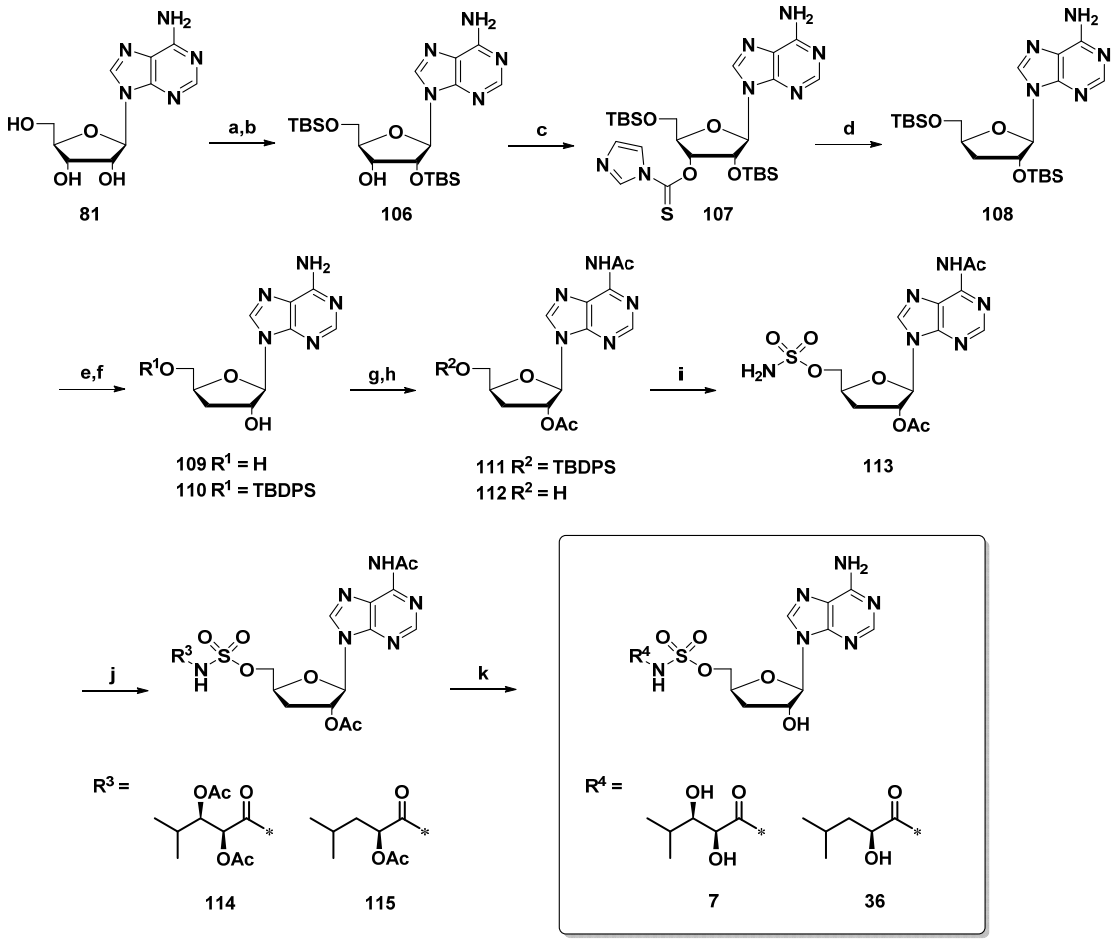


Scheme 13. Synthesis of 2-iodoadenylate analogues.

Reagents & conditions: (a) corresponding acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h, 45~80%; (b) 0.02 M NaOMe in MeOH, r.t, 2 h, 68~85%.

Synthesis of 3'-deoxyadenylate analogues is illustrated in **scheme 14**. 3'-deoxyadenosine (**109**) was prepared from adenosine (**81**) in 5 steps. Selective *tert*-butyldimethylsilane (TBDMS) protection of 2' and 5'-hydroxyl group of adenosine provided compound **106**. Barton-McCombie deoxygenation reaction of 3'-hydroxyl group afforded compound **108** followed by removal of two TBDMS groups afforded compound **109**. 5'-*O*-sulfamoylated 3'-deoxyadenosine (**113**) was synthesized in 4 steps, selective *tert*-butyldiphenylsilane (TBDPS) protection of 5'-hydroxyl group, acetylation, selective deprotection of TBDPS group followed by sulfamoylation provided compound **113**. Amide coupling with

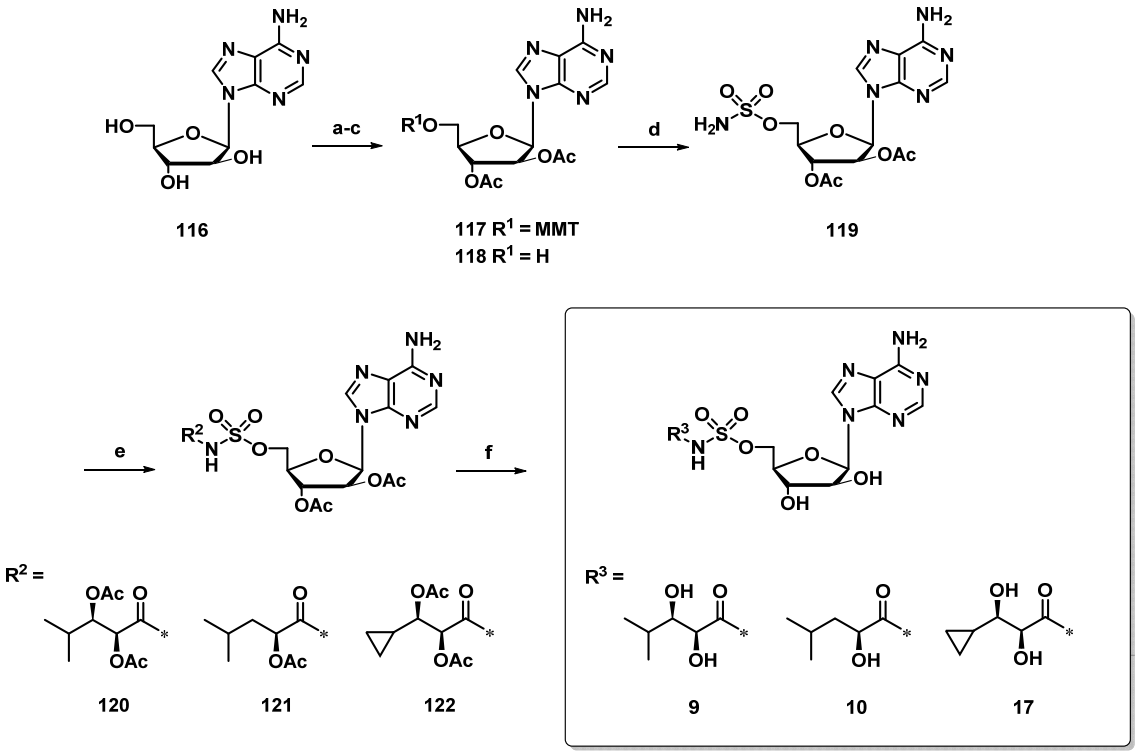
corresponding protected chiral acids afforded intermediates **114** and **115** and removal of acetyl groups yielded compounds **7** and **36**, respectively.



Scheme 14. Synthesis of 3'-deoxyadenylate analogues.

Reagents & conditions: (a) TBSCl, pyridine, r.t, 48 h; (b) TEA, MeOH, r.t, overnight, 55% in 2 steps ; (c) thiocarbonylimidazole, DMAP, CH_2Cl_2, r.t, overnight, 93%; (d) *n*-Bu_3SnH, AIBN, toluene, reflux, 6 h, 91%; (e) TBAF, THF, 0 ^{o}C to r.t, overnight, 87%; (f) TBDPSCl, TEA, DMF, DMAP, r.t, overnight, 73%; (g) Ac_2O, pyridine, 60 ^{o}C, 8 h, 93%; (h) TBAF, THF, 0 ^{o}C, 1 h, 89%; (i) NH_2SO_2Cl, NaH, anhyd. THF, 0 ^{o}C to r.t, 5 h, 75%; (j) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h, 65% for **114**, 69% for **115**; (k) 0.02 M NaOMe in MeOH, r.t, 2 h, 65% for **7**, 30% for **36**.

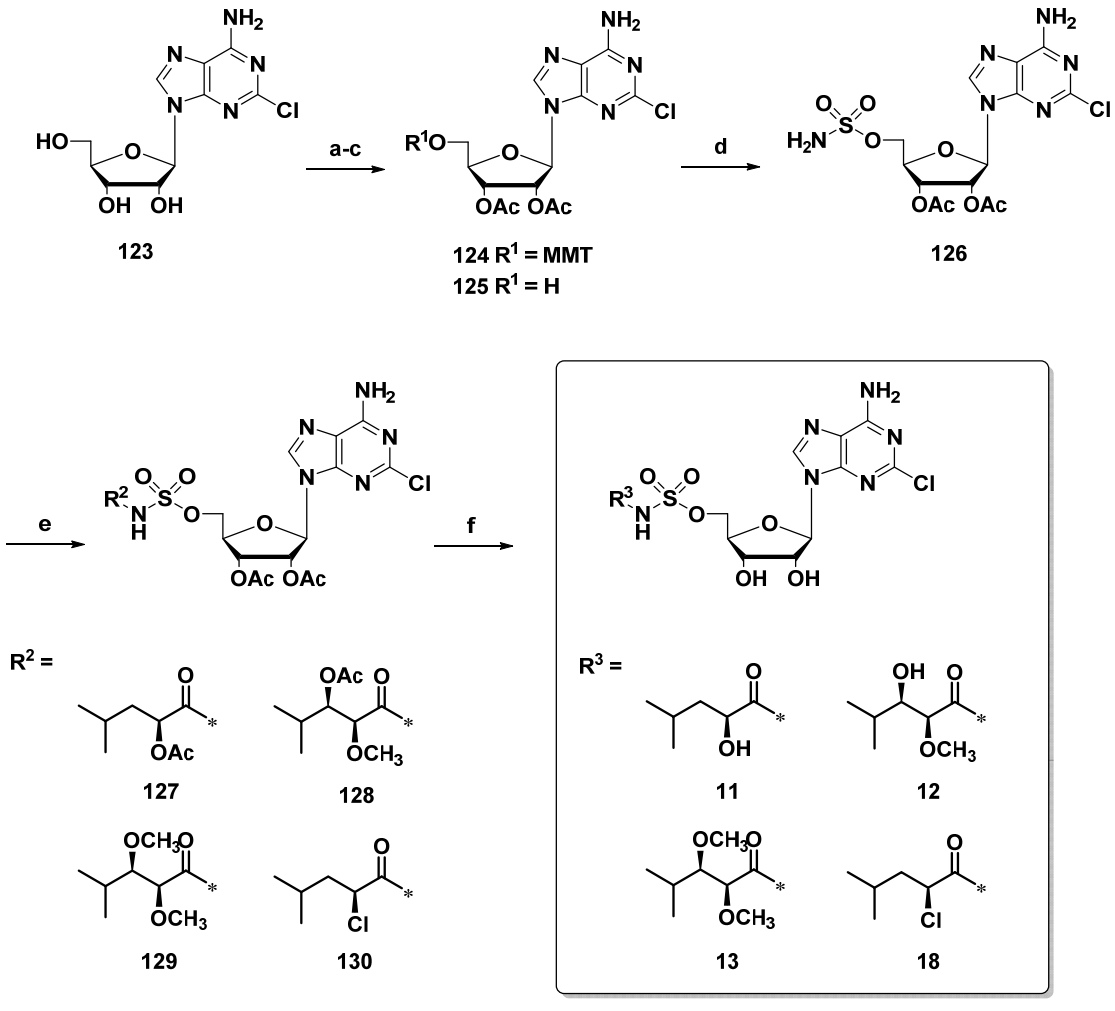
Synthesis of adenine arabinose analogues was carried out as illustrated in **scheme 15**. 5'-*O*-sulfamoylated intermediate **119** was prepared from commercially available adenine 9-β-D-arabinofuranoside (**116**) as described in **scheme 5**. Peptide coupling with prepared side chains provided compounds **120-122** followed by deprotection of acetyl groups afforded final compounds **9**, **10** and **17**, respectively.



Scheme 15. Synthesis of adenine arabinose analogues.

Reagents & conditions: (a) MMTCl, anhyd. pyridine, anhyd. DMF, 0 °C to r.t, 24 h; (b) acetic anhydride, TEA, DMAP, CH_3CN, 0 °C to r.t, 4 h; (c) 80% aq. AcOH, r.t, 12 h; (d) i) NaH, anhyd. THF, 0 °C, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 °C to r.t, 5 h; (e) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (f) 0.02 M NaOMe in MeOH, r.t, 2 h.

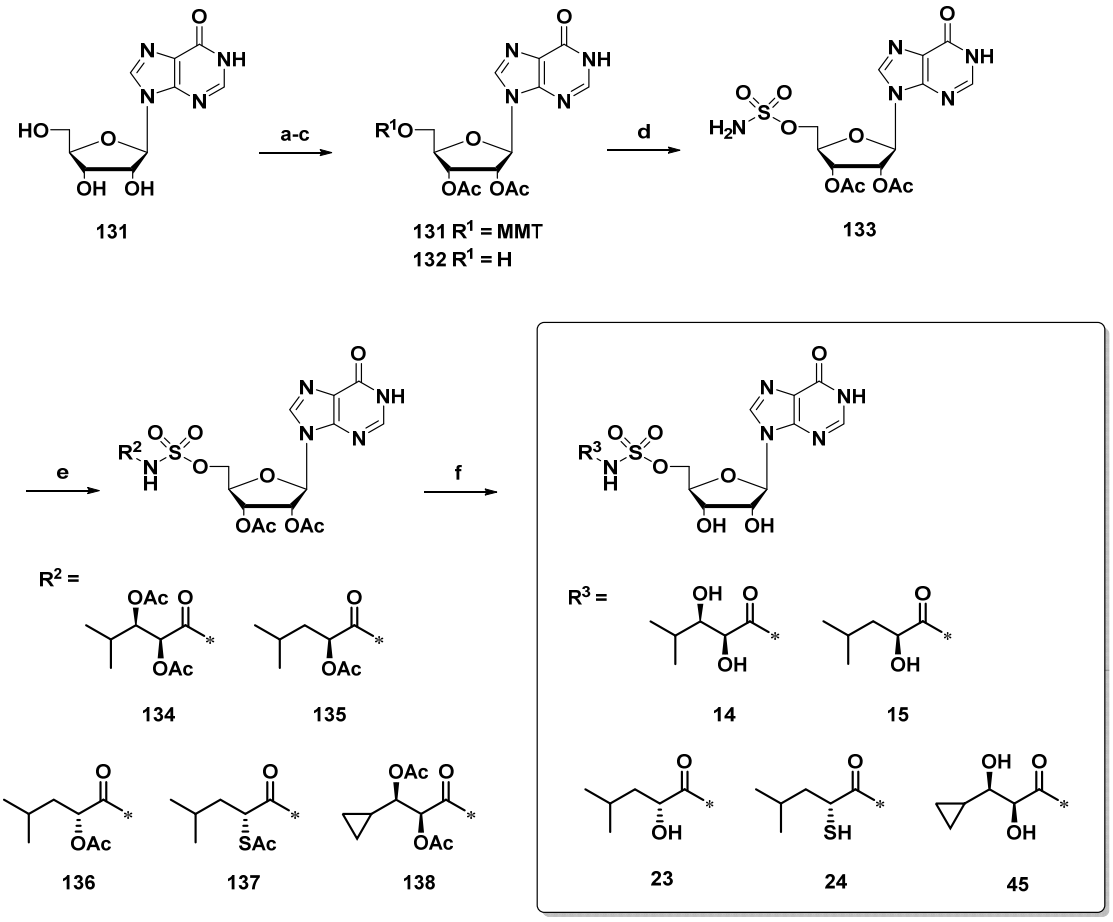
Synthesis of 2-chloroadenylate analogues was synthesized as shown in **scheme 16**. 5'-*O*-sulfamoylated intermediate **126** was prepared from commercially available 2-chloroadenosine (**123**) in 4 steps as described in **scheme 5**. Peptide coupling with prepared side chains provided compounds **127-129** followed by deprotection of acetyl groups afforded final compounds **11-13**, respectively. An amide coupling reaction between compound **126** and commercially available (*S*)-2-chloro-4-methylpentanoic acid followed by deprotection of the acetyl groups provided the final compound **18**.



Scheme 16. Synthesis of 2-chloroadenylate analogues.

Reagents & conditions: (a) MMTCl, anhyd. pyridine, anhyd. DMF, 0 °C to r.t, 24 h; (b) acetic anhydride, TEA, DMAP, CH_3CN, 0 °C to r.t, 4 h; (c) 80% aq. AcOH, r.t, 12 h; (d) i) NaH, anhyd. THF, 0 °C, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 °C to r.t, 5 h; (e) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (f) 0.02M NaOMe in MeOH, r.t, 2 h.

Synthesis of inosine analogues is shown in **scheme 17**. 5'-*O*-sulfamoylated intermediate **133** was prepared in 4 steps following the same route described in **scheme 5**. DCC coupling with corresponding protected chiral acids provided intermediates **134-138**, then removal of protection groups yielded final compounds **14**, **15**, **23**, **24** and **45**, respectively.

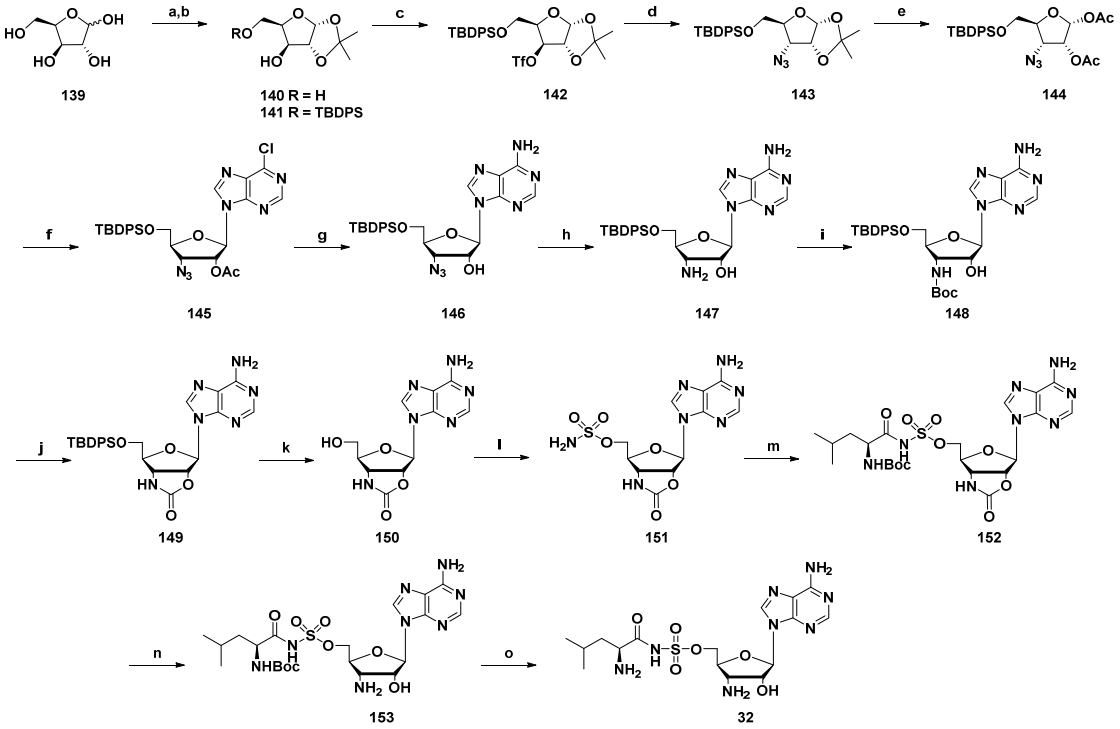


Scheme 17. Synthesis of inosine analogues.

Reagents & conditions: (a) MMTCl, anhyd. pyridine, anhyd. DMF, 0 °C to r.t, 24 h; (b)

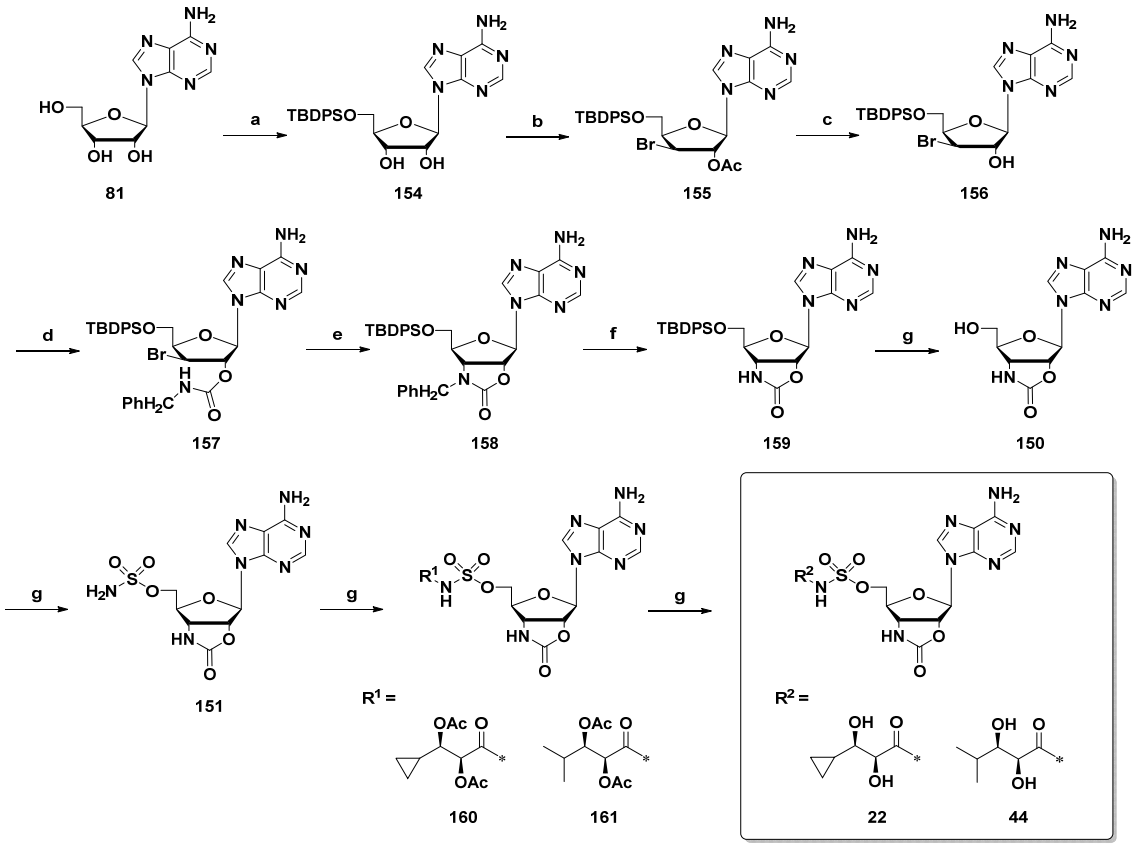
acetic anhydride, TEA, DMAP, CH_3CN, 0 ºC to r.t, 4 h; (c) 80% aq. AcOH, r.t, 12 h; (d) i) NaH, anhyd. THF, 0 ºC, 1 h, ii) NH_2SO_2Cl, anhyd. THF, 0 ºC to r.t, 5 h; (e) chiral acid, DCC, DMAP, anhyd. CH_2Cl_2, r.t, 12 h; (f) 0.02M NaOMe in MeOH, r.t, 2 h.

Synthesis of 3'-amino-3'-deoxyadenylate analogue **32** is shown in **scheme 18**. Isopropylidene protection of 1' and 2'-hydroxyl group and TBDPS protection of 5'-hydroxyl group of commercially available D-xylose provided compound **141**. 3'-Triflate group was substituted with azido group with inversion of stereochemistry to afford compound **143**. Isopropylidene group was removed and acetyl groups were introduced simultaneously at the same condition to give compound **144**. Condensation reaction of compound **144** with 6-chloropurine provided desired β-form **145** with 50% yield. After removal of acetyl group, azido group was reduced to give compound **147**. *N*-Boc protection followed by cyclization under basic condition provided compound **149**. 5'-*O*-sulfamoylated intermediate **151** was prepared by TBDPS deprotection and sulfamoylation of compound **149**. An amide coupling with commercially avaiable *N*-Boc leucine afforded compound **152** followed by removal of carbamate group and *N*-Boc protection group yielded the final compound **32**.



Scheme 18. Synthesis of compound **32**.

(a) $CuSO_4$, Me_2CO, H_2SO_4, r,t, 24 h, 30%; (b) TBDPSCl, imidazole, DMAP, DMF, 0 ºC to r.t, overnight, 57%; (c) $(CF_3SO_2)_2O$, pyridine, MC, -10 ºC, 30 min, 80% (d) NaN_3, pyridine, DMF, r.t, 7 days, 65%; (e) Ac_2O, AcOH, H_2SO_4, 0 ºC, 1 h, 95%; (f) (i) 6-chloropurine, HMDS, $(NH_4)_2SO_4$, reflux, 24 h, (ii) TMSOTf, anhyd. CH_2Cl_2, r.t, 68 h, 50%; (g) NH_3 2 M in MeOH, r.t, 24 h, 77%; (h) Lindlar catalyst, H_2, MeOH, r.t, overnight; (i) Boc_2O, Na_2CO_3, $THF:H_2O$ = 4:1, 0 ºC to r.t, overnight, 53% (j) 2 N NaOH, THF:MeOH = 1:1, r.t, overnight, 60%; (k) TBAF, THF, 0 ºC to r.t, overnight, 99%; (l) NH_2SO_2Cl, NaH, anhyd. THF, 0 ºC to r.t, 5 h, 82%; (m) *N*-Boc-Leu, DCC, DMAP, anhyd. THF, r.t, 12 h, 64%; (n) 1 N NaOH, THF, r.t, 48 h; (o) 1 N HCl in MeOH, r.t, overnight.



Scheme 19. Synthesis of 3'-amino-3'-deoxyadenylate analogues.

(a) TBDPSCl, dry pyridine, r.t, 40 h, 79%; (b) $AcOCMe_2COBr$, ACN, 0.8 eq.H_2O, 0'C, 5 h, 78%; (c) K2CO3, MeOH, 0 ^oC, 48 h, 81%; (d) BnNCO, toluene, 90'C, overnight, 80%; (e) NaH, anhyd.THF, -20 ^oC, 1 h, 99%; (f) KBr, Oxone, ACN, 70 ^oC, 24 h, 63%; (g) TBAF, anhyd. THF, 0 ^oC to r.t, 2 h, 90%; (h) NH_2SO_2Cl, NaH, anhyd. DMF, 0 ^oC to r.t, 12 h, 76%; (i) chiral acid, DCC, DMAP, anhyd. THF:DMF, r.t, 12 h, 44% for **160**, 55% for **161** (j) 0.02 M NaOMe, r.t, 4 h, 90% for **22**, 87% for **44**.

Synthesis of 3'-amino-3'-deoxyadenylate analogues is illustrated in **scheme 19**. Compound **158** was prepared in 5 steps from adenosine (**81**) by following a previously reported procedures.[41] Oxidative debenzylaion of *N*-benzyl amide was carried out by a bromo radical formed through the oxidation of potassium bromide under mild condition.[42] Removal of 5'-TBDPS group and sulfamoylation provided 5'-*O*-sulfamoylated intermediate **151**. Amide coupling with corresponding protected chiral acids afforded

compounds **160** and **161** followed by deprotection of acetyl groups yielded final compounds **22** and **44**, respectively.

2.2. Biological Activity

To evaluate the effect of the synthesized compounds on cellular mTORC1 pathway, we first determined leucine-induced phosphorylation of S6Kinase (S6K) in HEK293 cells by immunoblots. For a primary screening, we pretreated HEK293 cells with compounds **2-45** at one fixed concentration (200 μM) as well as with compound **1**, rapamycin (100 nM) and leucinol (800 μM) for comparison, and then activated mTORC1 by treating the cells with leucine for 10 min. As shown in **Figure 16**, leucine induced phosphorylation of S6K, demonstrating an intense band that corresponds to pS6K whereas rapamycin and compound **1** inhibited S6K phosphorylation. Among the tested compounds, compounds **4** and **5** appeared to show more potent inhibition of S6K phosphorylation than compound **1**, while compounds **1** and **2** appeared to inhibit S6K phosphorylation to a similar extent as compound **1**.

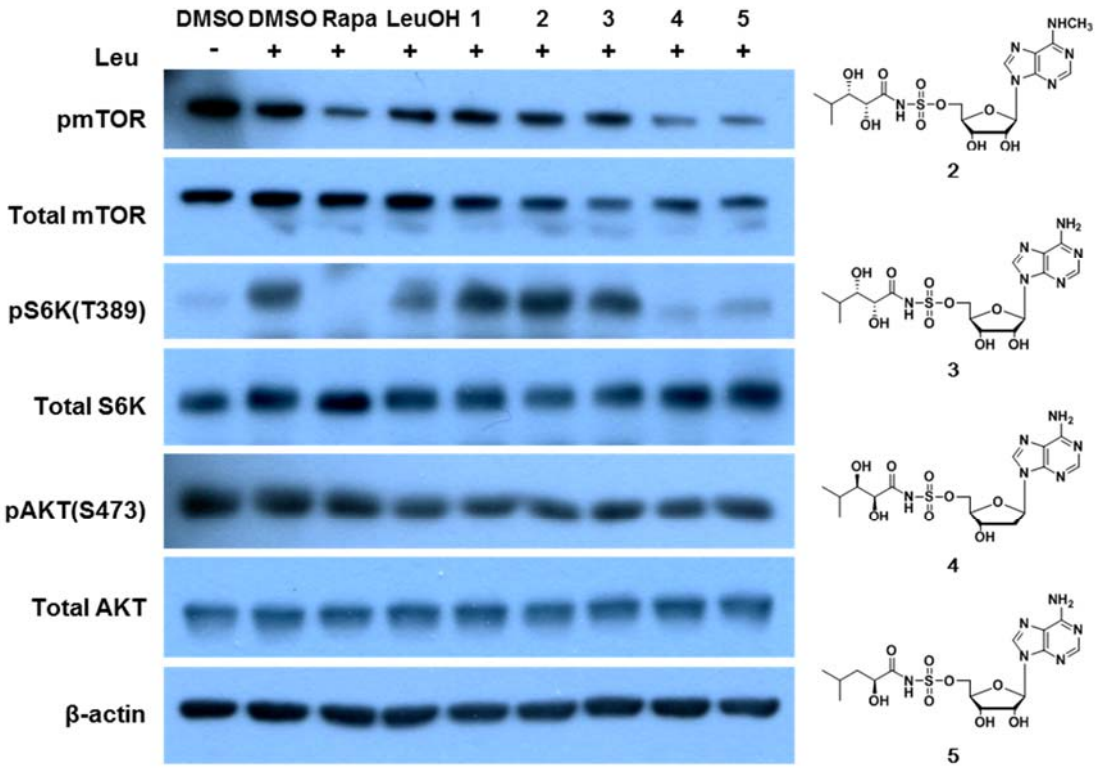


Figure 16. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **2-5** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we focused on the compounds **4** and **5** and confirmed the dose-dependent inhibition of S6K phosphorylation (**Figure 17**). Compound **4** inhibited S6K phosphorylation in a dose-dependent manner, while compound **5** showed dose-dependent inhibition except at 200 μM concentration. When 2'-hydroxyl group or β-hydroxyl group of the leucyl side chain is removed, the activity of mTORC1 inhibition enhanced than compound **1** in both case. According to the result, 2'-hydroxyl group is not necessary to mTORC1 inhibition and also, (*S*)-2-hydroxy-4-methylpentanoyl side chain has favorable effect on the mTORC1 inhibition.

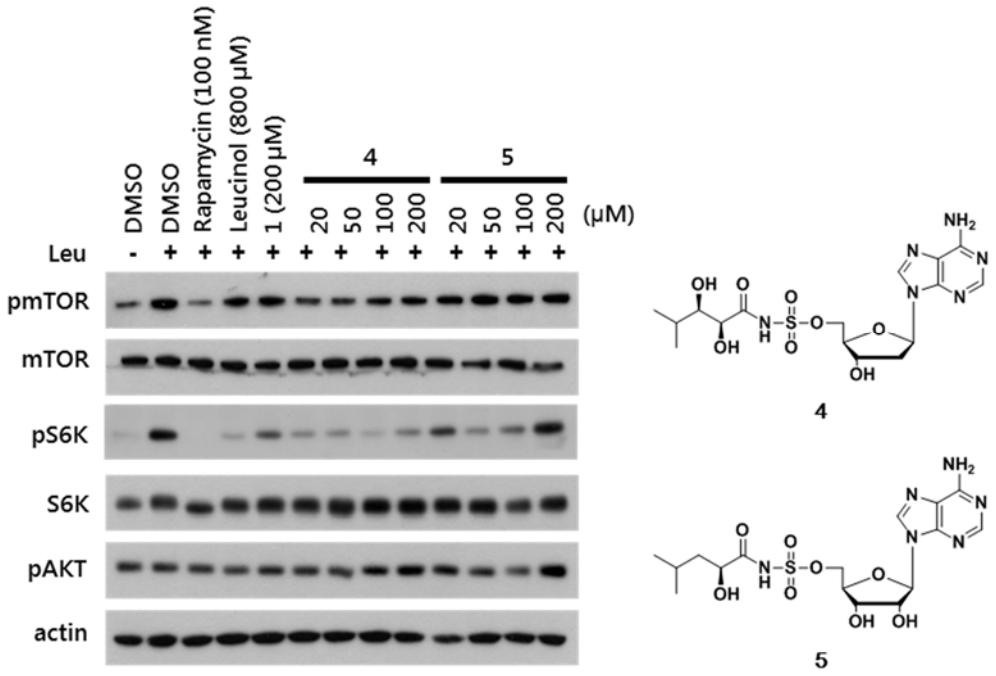


Figure 17. Dose dependent inhibition of mTORC1 activation by compounds **4** and **5**.

Next, we examined the inhibition of mTORC1 activation with compounds **6** and **7** (**Figure 18**). Compound **6** appeared to inhibit S6K phosphorylation to a similar extent as compound **1**, while compound **7** did not inhibit S6K phosphorylation at the same concentration. When 2-iodo group is introduced in the adenine, the potency of mTORC1 inhibition maintained. However, when 3'-hydroxyl group is removed, the potency of mTORC1 inhibition decreased, suggesting that 3'-hydroxyl group is essential in binding interaction in LRS catalytic site.

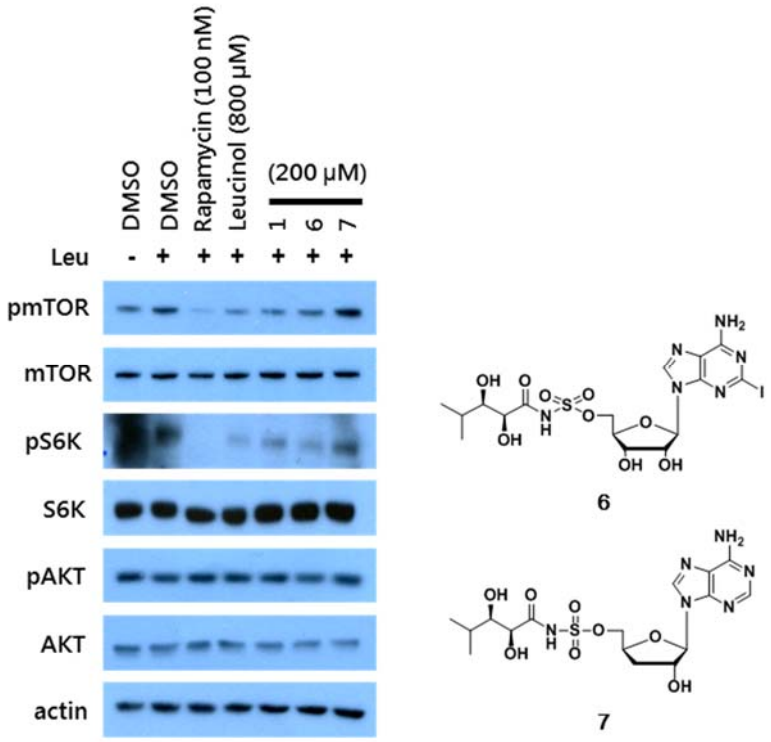


Figure 18. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **6** and **7** at fixed concentrations.

Next, we determined the effects of compounds **8-11** on leucine-induced mTORC1 activation using the immunoblotting method. As shown in **Figure 19**, compounds **8** and **10** maintained the activity of mTORC1 inhibition to a same extent as compound **1** but, compounds **9** and **11** lost the activity. To our surprise, the inhibition of S6K phosphorylation maintained when both α, β-hydroxyl group were removed. When adenylate group is substituted with adenine arabinose, compound **10** which has α-hydroxyl group in leucyl side chain showed more potent inhibition than compound **9** with both α- hydroxyl and β-hydroxyl group, which is in agreement with previous reported observation.[30] Also, when 2-chloro group in introduced in adenine, it lost the activity at some extent, suggesting that 2-iodo group is crucial in the binging interaction in catalytic

site of LRS.

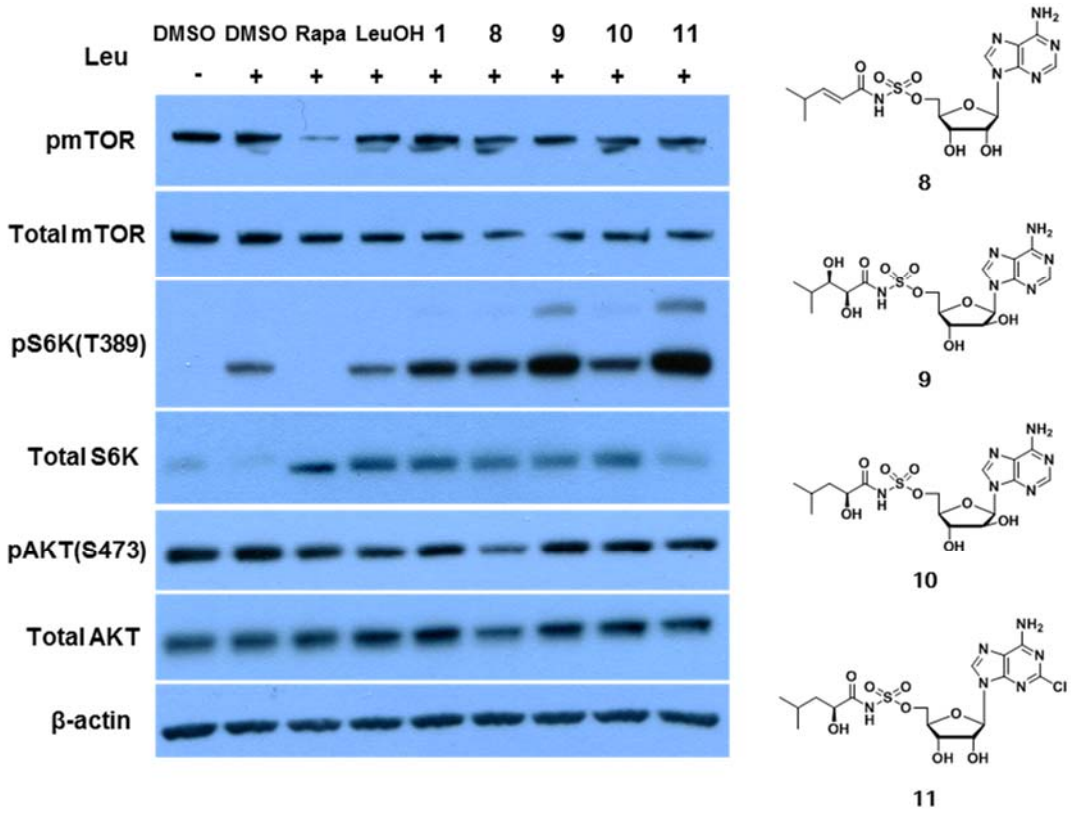


Figure 19. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **8-11** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we examined the inhibition of mTORC1 activation with compounds **12-15**. As shown in **Figure 20**, compound **12** appeared to inhibit S6K phosphorylation to a similar extent as compound **1**, while compounds **13-15** demonstrated more potent inhibition than compound **1**. According to the result, α-methoxy group has a favorable effect on the inhibition of S6K phosphorylation and α, β-methoxy group further enhanced the inhibition of S6K phosphorylation. Also, inosine derivatives showed potent inhibition of

mTORC1 activation in both compounds **14** and **15**, which has α-hydroxyl group and α, β-hydroxyl group, respectively. Based on the band intensity of pS6K, compound **15** appeared slightly more potent than compound **14**, which is again in agreement with previous report.[30]

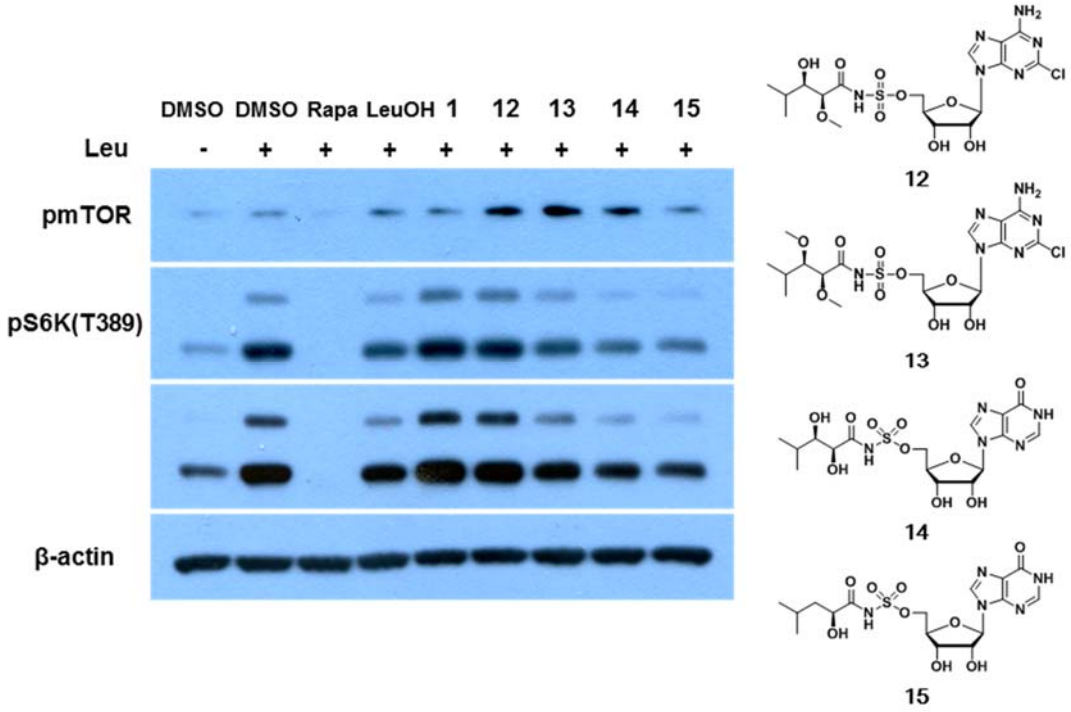


Figure 20. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **12**-**15** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we examined the inhibition of mTORC1 activation with compounds **16**-**18** (**Figure 21**). Based on band intensities of pS6K, when agrocin side chain is introduced, the inhibition of S6K phosphorylation enhanced slightly, suggesting structurally more rigid cyclopropyl group has favorable effect on the activity of mTORC1 inhibition. In the case of compound **18**, when α-hydroxyl group is substituted with α-chloro group, intensity of inhibition of S6K phosphorylation increased extensively. It is interesting that

hydrophobic chloro group instead of hydrophilic hydroxyl group has favorable effect on the binding affinity in catalytic site of LRS.

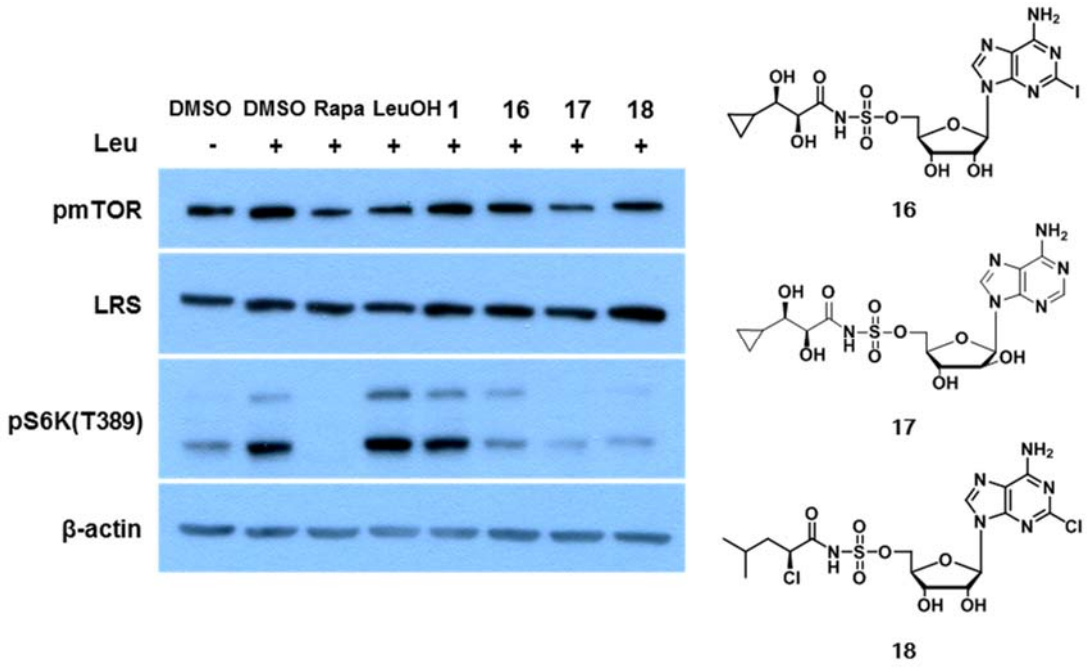


Figure 21. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **16-18** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we determined the effects of compounds **19-25** on leucine-induced mTORC1 activation using the immunoblotting method. As shown in **Figure 22**, compounds **20-22** appeared to inhibit mTORC1 activation to a similar extent as compound **1**, while compounds **19**, **23-25** did not inhibit mTORC1 activation. When 3-isopropyl-1-methyl-1H-pyrazole-5-carboxylic acid group is coupled as a sulfonamide group with adenylate and 2-iodoadenylate, the activity of mTORC1 inhibition maintained. Also, compound **22** which has both agrocin side chain and 3'-deoxy-3'-amino adenylate structure showed

similar extent of inhibition of mTORC1 activation. Inosine derivatives with (*R*)-hydroxyl group (**23**) or (*S*)-thiol group (**24**) instead of (*S*)-hydroxyl group at the α-position adversely affected on the activity of mTORC1 inhibition.

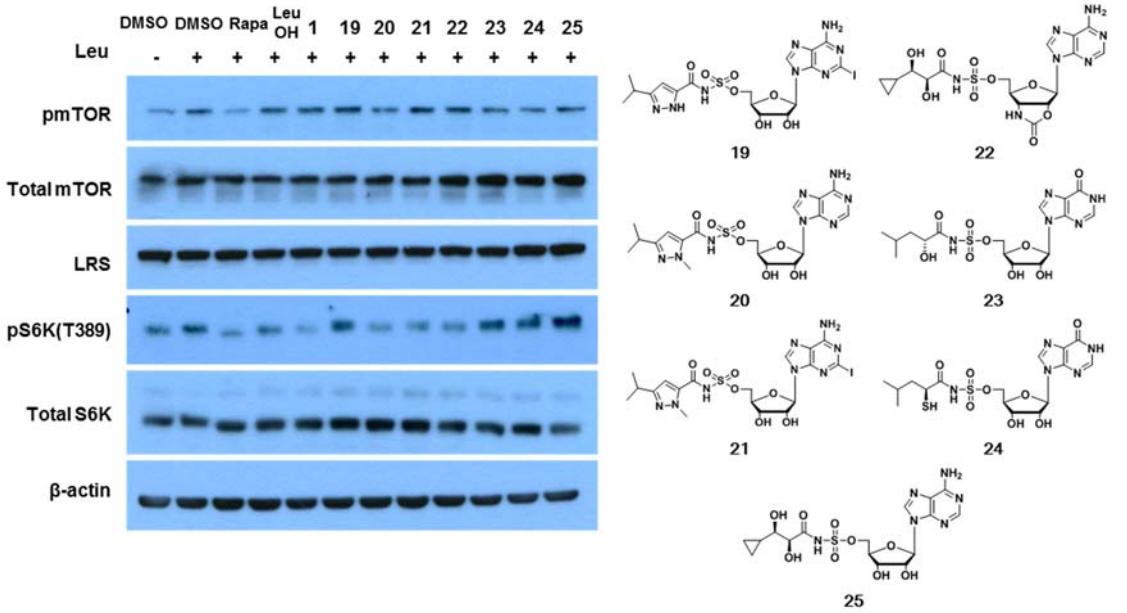


Figure 22. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **19-25** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we examined the inhibition of mTORC1 activation with compounds **26-31** (**Figure 23**). According to band intensities of pS6K, compounds **27**, **29** and **30** appeared to inhibit S6K phosphorylation to a similar extent as compound **1**, while compounds **26**, **28** and **31** showed less potent inhibition of S6K phosphorylation. When pyrazole group is introduced in leucyl side chain, only compound with 3-*t*-butyl pyrazole group (**27**) showed inhibition of mTORC1 activation, while compounds with 3-isopropyl (**26**) and 3-cyclopropyl pyrazole (**28**) did not showed activity. Also, compounds with (*R*)-hydroxyl

group (**29**) or (*S*)-thiol group (**30**) instead of (*S*)-hydroxyl group at the α-position maintained the activity, which is an opposite result with that of inosine derivatives. Compound **31** with *N*-methyl amino group instead of (*S*)-hydroxyl group slightly less potent inhibition than compound **1**.

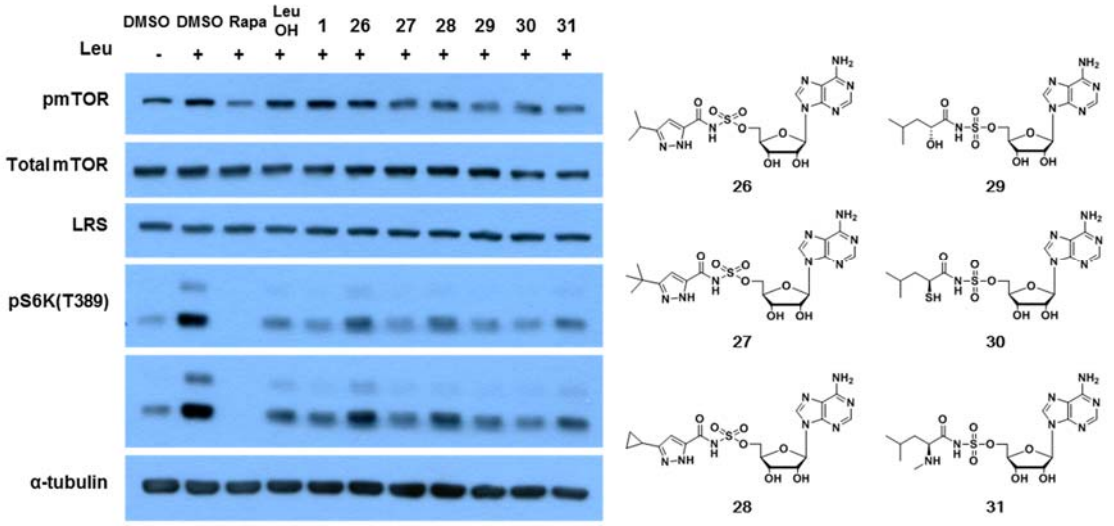


Figure 23. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **26-31** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we assessed the inhibition of mTORC1 activation with compounds **32-34**. As shown in **Figure 24**, all compounds showed less potent inhibition of S6K phosphorylation. Both 3'-deoxy-3'-aminoadenylate and 2'-deoxyadenylate with leucyl side chain did not inhibit S6K phosphorylation and 2'-deoxyadenylate with (2*R*,3*S*)-α,β-dihydroxyl side chain also lost activity.

Next, we examined the inhibition of mTORC1 activation with compounds **35-39**. As shown in **Figure 25**, all compounds did not show more potent inhibition of S6K

phosphorylation than compound **1**. Both N^6-methyladenylate and 3'-deoxyadenylate with α-hydroxyl side chain did not inhibit mTORC1 activation and also, 2'-deoxyadenylate with α-hydroxyl group and α-methoxy-β-hydroxyl group did not show potent inhibition than compound **1**.

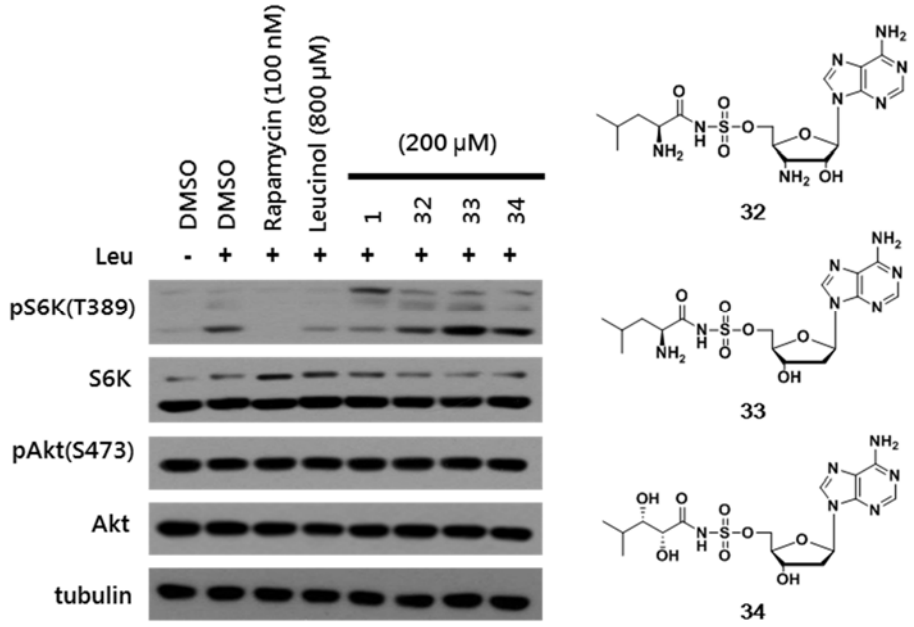


Figure 24. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **32**-**34** at fixed concentration.

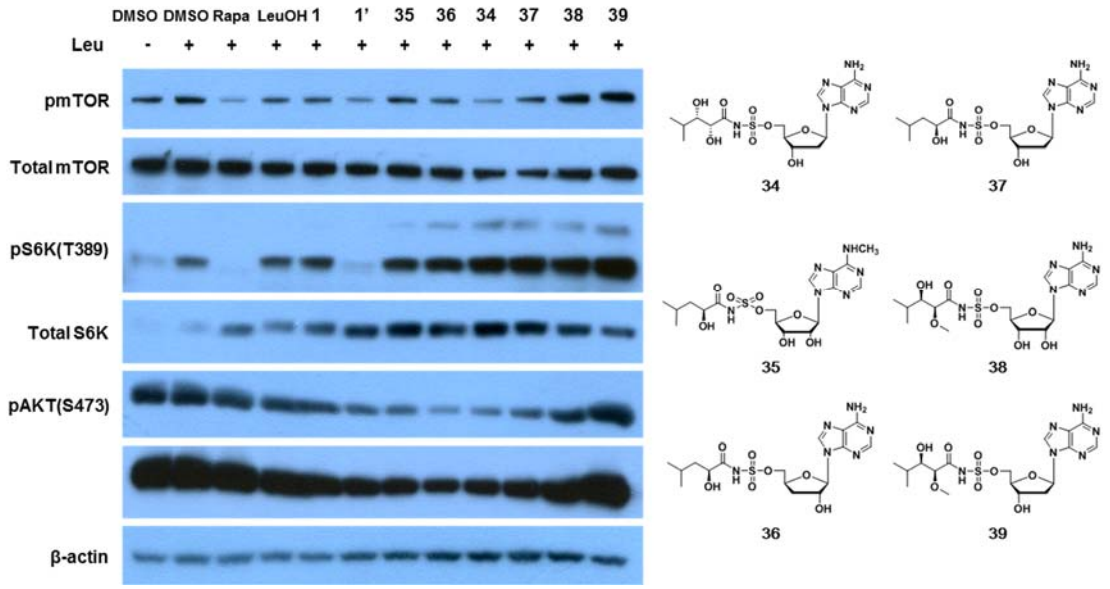


Figure 25. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **35**-**39** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

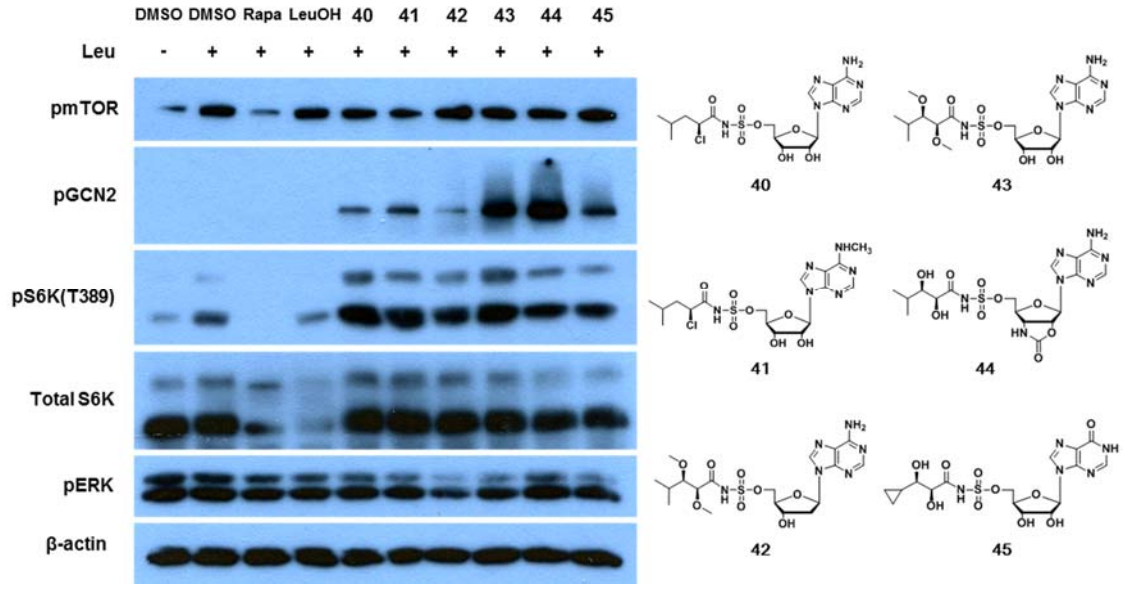


Figure 26. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds **40**-**45** at fixed concentrations (200 μM). Rapamycin was treated at 100 nM, leucinol was at 800 μM.

Next, we assessed the inhibition of mTORC1 activation with compounds **40**-**45** (**Figure 26**). According to the band intensities of pS6K, all compounds did not inhibit S6K phosphorylation when compared to leucinol. Compounds **40** and **41** with α-chloro group adversely affected on the activity of mTORC1 inhibition, which is an opposite result with that of 2-chloroadnylate (**18**). Compounds with α, β-dimethoxy group (**42**, **43**) did not show mTORC1 inhibition and when agrocin side chain is introduced in inosine derivative (**45**), the activity also did not improved.

3. Conclusion

We have developed leucyladenylate sulfamate derivatives that directly interact with LRS to inhibit the mTORC1 pathway.[30] Based on these previous findings, we synthesized a library of leucyladenylate sulfamates by modifying adenine (group 1), ribose (group 2) and leucine moieties (group 3) and evaluated their biological activity. When we tested these compounds by using immunoblots, we found that group 1 compounds with a lipophilic group generally showed good activity against mTORC1, while 2-iodoadenylate derivatives were most active. Inosine derivatives also showed good inhibition against mTORC1 activity, which is a same trend with 2-iodoadenylate derivatives. On the other hand, any modifications in the ribose moiety (group 2) lead to the loss of activity when compared to the most potent previously reported compound.[30] Among the adenylate derivatives, compound **5** with α-hydroxyl group was most potent. However, among the 2'-deoxyadeylate derivatives, compound **4** with α, β-hydroxyl group was most active. In the case of arabinose derivatives, compound with agrocin side chain (**17**) showed potent

activity. Among the group 3 compounds, generally replacement of the α-amino group of leucine with a hydroxyl group significantly enhanced inhibitory activity against mTORC1, while introduction of an additional hydroxyl group also resulted in good inhibitory effect. According to the biological activity result, the potency of inhibition of mTORC1 activation depends on the combination of adenine, ribose ring and side chain structure.

4. Experimental

4.1. General Experimental

All chemical reagents were commercially available. Melting points were determined on a Büchi Melting Point B-540 apparatus and are uncorrected. Silica gel column chromatography was performed on silica gel 60, 230-400 mesh, Merck. Nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra were recorded on JEOL JNM-LA 300 [300 MHz (^{1}H), 75 MHz (^{13}C)] and Bruker Avance 400 MHz FT-NMR [400 MHz (^{1}H), 100 MHz (^{13}C)] spectrometers. Chemical shifts are reported in ppm units with Me_4Si as a reference standard. Mass spectra were recorded on a VG Trio-2 GC-MS and 6460 Triple Quad LC/MS. All final compounds were purified to >95% purity, as determined by high-performance liquid chromatography (HPLC). HPLC was performed on an Agilent 1120 Compact LC (G4288A) instrument using an Agilent Eclipse Plus C18 column (4.6 x 250 mm, 5 μm) and a Daicel Chiralcel OD-H column (4.6 x 250 mm, 5 μm).

4.2. Chemical spectra

4.2.1. ((2*R*,3*S*,5*R*)-5-(6-amino-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoyl)sulfamate (4)

^{1}H NMR (500 MHz, CD_3OD): δ 8.49 (s, H_8), 8.17 (s, H_2), 6.49 (t, J = 6.80 Hz, $H_{1'}$), 4.63 (t, J = 2.75 Hz, $H_{4'}$), 4.30-4.24 (m, $H_{5'}$, $H_{5''}$), 4.19 (q, J = 2.80 Hz, H_α), 4.10 (s, $H_{3'}$), 3.48 (d, 11.60 Hz, H_β), 2.76 (qd, J = 6.30, 0.75 Hz, $H_{2'}$), 2.47 (qd, J = 6.10, 3.05 Hz, $H_{2''}$), 1.90-1.83 (m, H_ν), 1.01 (d, J = 6.60 Hz, 3H), 0.94 (d, J = 6.75 Hz, 3H), ^{13}C NMR (125 MHz, CD_3OD): δ 182.3, 158.0, 154.6, 151.1, 142.0, 120.8, 87.4, 86.4, 80.5, 75.5, 73.7, 70.3, 42.3, 32.8, 20.6, 20.3; MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{24}N_6O_8S$: 461.1454; found: 461.1443.

4.2.2 ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl)sulfamate (5)

^{1}H NMR (500 MHz, CD_3OD): δ 8.50 (s, H_8), 8.19 (s, H_2), 6.08 (d, J = 5.35 Hz, $H_{1'}$), 4.64 (t, J = 7.90 Hz, $H_{2'}$), 4.38 (t, J = 3.80 Hz, $H_{3'}$), 4.35-4.25 (m, $H_{4'}$, $H_{5'}$, $H_{5''}$), 4.03 (dd, J = 9.15, 3.25 Hz, H_α), 1.65-1.43 (m, 3H), 0.91 (t, J = 6.75 Hz, 6H), ^{13}C NMR (125 MHz, CD_3OD): δ 184.2, 158.0, 154.7, 151.5, 142.1, 120.8, 90.1, 85.1, 76.9, 73.8, 72.8, 69.9, 46.4, 26.4, 24.8, 22.6; MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{24}N_6O_8S$: 461.1454; found: 461.1445.

4.2.3. ((2*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-2-

yl)methyl ((2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoyl)sulfamate (7)

^{1}H NMR (500 MHz, CD_3OD): δ 8.46 (s, H_8), 8.18 (s, H_2), 5.99 (d, J = 1.55 Hz, $H_{1'}$), 4.70-4.66 (m, $H_{4'}$), 4.65-4.64 (m, $H_{2'}$), 4.43 (dd, J = 11.25, 2.35 Hz, $H_{5'}$), 4.26 (dd, J = 11.25, 3.25 Hz, $H_{5''}$), 4.11 (s, H_α), 3.51 (d, J = 8.10 Hz, H_β), 2.40 (qd, J = 9.00, 5.65 Hz, $H_{3'}$), 2.11 (qd, J = 7.15, 4.45 Hz, $H_{3''}$), 1.00 (d, J = 6.65 Hz, 3H), 0.94 (d, J = 6.75 Hz, 3H), ^{13}C NMR (125 MHz, CD_3OD): δ 182.4, 158.0, 154.5, 150.8, 141.8, 121.0, 93.7, 80.9, 80.4, 77.8, 75.5, 70.8, 35.3, 32.8, 20.6, 20.3; MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{24}N_6O_8S$: 461.1454; found: 461.1452.

4.2.4. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-chloro-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl)sulfamate (11)

^{1}H NMR (500 MHz, CD_3OD): δ 8.37 (s, 1H, Ar, 8H), 5.99 (d, 1H, CH, 1'H), 4.58 (t, 1H, CH, 2'H), 4.43 (qd, 2H, CH_2, 5'H), 4.36 (t, 1H, CH, 3'H), 4.28 (q, 1H, CH, 4'H), 4.02 (dd, 1H, CH), 1.81 (m, 1H, CH), 1.48 (m, 2H, CH_2), 0.92 (s, 3H, CH_3), 0.90 (s, 3H, CH_3); MS (HR-ESI) *m/z* $[M + H]^+$ found 495.1068.

4.2.5. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-chloro-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-3-hydroxy-2-methoxy-4-methylpentanoyl)sulfamate (12)

^{1}H NMR (500 MHz, CD_3OD): δ 8.48 (s, 1H, Ar, 8H), 6.01 (d, 1H, CH, 1'H), 4.59 (t, 1H, CH, 2'H), 4.39 (t, 1H, CH, 3'H), 4.32 (qd, 2H, CH_2, 5'H), 4.28 (q, 1H, CH, 4'H), 3.66 (d,

1H, CH), 3.47 (dd, 1H, CH), 3.36 (s, 3H, CH_3), 1.85 (m, 1H, CH), 0.97 (d, 3H, CH_3), 0.90 (s, 3H, CH_3) ^{13}C NMR (125 MHz, CD_3OD): δ 180.954, 158.805, 156.222, 152.858, 142.248, 119.843, 90.102, 86.499, 85.249, 79.804, 77.057, 72.887, 69.893, 59.398, 32.618, 20.667, 19.656; MS (HR-ESI) *m/z* $[M + H]^+$ found 525.1162.

4.2.6. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-chloro-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dimethoxy-4-methylpentanoyl)sulfamate (13)

^1H NMR (500 MHz, CD_3OD): δ 8.49 (s, 1H, Ar, 8H), 6.01 (d, 1H, CH, 1'H), 4.61 (t, 1H, CH, 2'H), 4.38 (m, 1H, CH, 3'H), 4.34 (m, 2H, CH_2, 5'H), 4.28 (m, 1H, CH, 4'H), 3.67 (d, 1H, CH), 3.40 (s, 3H, CH_3), 3.33 (s, 3H, CH_3), 3.21 (m, 1H, CH), 1.91 (m, 1H, CH), 0.97 (d, 3H, CH_3), 0.89 (s, 3H, CH_3), ^{13}C NMR (125 MHz, CD_3OD): δ 180.474, 158.866, 156.234, 152.898, 142.267, 119.850, 90.298, 90.072, 86.315, 85.342, 77.040, 72.984, 69.990, 62.143, 59.404, 32.074, 20.597, 19.667; MS (HR-ESI) *m/z* $[M + H]^+$ found 539.1287.

4.2.7. ((2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoyl)sulfamate (14)

^1H NMR (500 MHz, CD_3OD): δ 8.42 (s, 1H, Ar, 2H), 8.04 (s, 1H, Ar, 8H), 6.05 (d, 1H, CH, 1'H), 4.62 (t, 1H, CH, 2'H), 4.38 (t, 1H, CH, 3'H), 4.18 (qd, 2H, CH_2, 5'H), 4.28 (m,

1H, CH, 4'H), 4.05 (d, 1H, CH), 3.50 (dd, 1H, CH), 1.85 (m, 1H, CH), 1.01 (d, 3H, CH_3), 0.94 (s, 3H, CH_3), ^{13}C NMR (125 MHz, CD_3OD): δ 182.163, 151.196, 149.741, 141.105, 126.111, 90.217, 85.208, 80.462, 77.066, 75.527, 72.887, 69.811, 32.873, 20.585, 20.445; MS (HR-ESI) *m/z* $[M + H]^+$ found 478.1230.

4.2.8. ((2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl)sulfamate (15)

^1H NMR (500 MHz, CD_3OD): δ 8.44 (s, 1H, Ar, 8H), 8.05 (s, 1H, Ar, 2H), 6.06 (d, 1H, CH, 1'H), 4.65 (t, 1H, CH, 2'H), 4.37 (m, 1H, CH, 3'H), 4.31 (m, 2H, CH_2, 5'H), 4.28 (m, 1H, CH, 4'H), 3.98 (dd, 1H, CH), 1.82 (m, 1H, CH), 1.54 (m, 1H, CH_2), 1.43 (m, 1H, CH_2), 0.91 (t, 6H, CH_3), ^{13}C NMR (125 MHz, CD_3OD): δ 183.395, 160.102, 150.339, 147.600, 140.740, 125.434, 89.557, 84.605, 76.233, 73.066, 72.219, 69.036, 45.601, 25.756, 24.037, 21.948; MS (HR-ESI) *m/z* $[M + H]^+$ found 462.1266.

4.2.9. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-2-chloro-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((*S*)-2-chloro-4-methylpentanoyl)sulfamate (18)

^1H NMR (500 MHz, CD_3OD): δ 8.46 (s, 1H, Ar, 8H), 6.01 (d, J = 5.95 Hz, 1H, CH, 1'H), 4.62 (t, J = 5.25 Hz, 1H, CH, 2'H), 4.36 (dd, J = 4.7, 3.15 Hz, 1H, CH, 3'H), 4.30 (m, 2H, CH_2, 5'H), 4.28 (m, 1H, CH_2, 4'H), 4.22 (dd, J = 8.3, 6.05 Hz, 1H, CH), 1.78 (m, 2H, CH_2), 1.71 (m, 1H, CH), 0.91 (dd, J = 23.55, 6.3 Hz, 6H, CH_3); MS (HR-ESI) *m/z*

$[M + H]^+$ found 513.0705.

4.2.10. ((3a*R*,4*S*,6*R*,6a*R*)-6-(6-amino-9H-purin-9-yl)-2-iminohexahydrofuro[3,4-d]oxazol-4-yl)methyl ((2*S*,3*R*)-3-cyclopropyl-2,3-dihydroxypropanoyl)sulfamate (22)

^{1}H NMR (500 MHz, CD_3OD): δ 8.68 (s, 1H, Ar, 8H), 8.58 (s, 1H, Ar, 2H), 6.34 (s, 1H, CH, 1'H), 4.48 (t, J = 3.5, 1H, CH, 2'H), 4.43 (d, J = 2.3, 1H, CH, 3'H), 4.26 (m, 2H, CH_2, 5'H), 4.20 (dd, J = 11.15, 3.75, 1H, CH, 4'H), 4.00 (m, 1H, CH), 3.09 (d, J = 8.8, 1H, CH), 2.71 (dd, J = 8.97, 2.55, 1H, CH), 1.14 (m, 1H, CH), 0.57 (m, 1H, CH), 0.49 (m, 1H, CH), 0.38 (m, 1H, CH), 0.30 (m, 1H, CH); MS (FAB) *m/z* 500 (M+H).

4.2.11. ((2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl ((*R*)-2-hydroxy-4-methylpentanoyl)sulfamate (23)

^{1}H NMR (500 MHz, CD_3OD): δ 8.31 (s, 1H, Ar, 8H), 8.05 (s, 1H, Ar, 2H), 6.27 (s, 1H, CH, 1'H), 4.80 (s, 1H, CH, 2'H), 4.71 (t, J = 1.6, 1H, CH, 3'H), 4.56 (dd, J = 13.9, 2.35, 1H, CH2, 4'H), 4.36 (m, 2H, CH_2, 5'H), 4.05 (d, J = 5.45, 1H, CH), 1.85 (m, 1H, CH), 1.52 (m, 2H, CH_2), 0.93 (dd, J = 11.95, 5.65 Hz, 6H, CH_3); MS (HR-ESI) *m/z* $[M + H]^+$ found 462.1305.

4.2.12. ((2*R*,3*S*,4*R*,5*R*)-3,4-dihydroxy-5-(6-oxo-1,6-dihydro-9H-purin-9-yl)tetrahydrofuran-2-yl)methyl ((*R*)-2-mercapto-4-methylpentanoyl)sulfamate (24)

^{1}H NMR (500 MHz, CD_3OD): δ 8.36 (s, 1H, Ar, 8H), 8.06 (s, 1H, Ar, 2H), 6.06 (s, 1H, CH, 1'H), 4.43 (m, 1H, CH, 2'H), 4.40 (m, 1H, CH, 3'H), 4.33 (m, 2H, CH2, 5'H), 4.29 (m, 1H, CH2, 4'H), 3.63 (m, 1H, CH), 1.78 (m, 1H, CH),), 1.68 (m, 1H, CH), 1.49 (m, 1H, CH), 0.90 (m, 6H, CH_3); MS (HR-ESI) *m/z* $[M + H]^+$ found 478.1056.

4.2.13. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-3-cyclopropyl-2,3-dihydroxypropanoyl)sulfamate (25)

^{1}H NMR (500 MHz, CD_3OD): δ 8.51 (s, H_8), 8.18 (s, H_2), 6.08 (d, J = 5.60 Hz, $H_{1'}$), 4.64 (t, J = 5.30 Hz, $H_{2'}$), 4.38 (dd, J = 8.20, 3.50 Hz, $H_{3'}$), 4.35-4.27 (m, H_β, $H_{5'}$, $H_{5''}$), 4.00 (d, J = 2.10 Hz, H_α), 3.11 (dd, J = 9.10, 2.00 Hz, $H_{4'}$), 1.17-1.11 (m, H_ν), 0.56-0.29 (m, 4H); MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{22}N_6O_9S$: 475.1247; found: 475.1245.

4.2.14. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((*R*)-2-hydroxy-4-methylpentanoyl)sulfamate (29)

^{1}H NMR (500 MHz, CD_3OD): δ 8.56 (s, 1H, Ar, 8H), 8.31 (s, 1H, Ar, 2H), 6.37 (s, 1H, CH, 1'H), 5.02 (d, J = 13.6, 1H, CH, 2'H), 4.75 (m, 2H, CH_2), 4.38 (t, J = 4.8, 1H, CH), 4.11 (d, J = 5.4, 1H, CH), 4.01 (m, 1H, CH), 1.84 (m, 1H, CH),), 1.51 (m, 1H, CH), 0.90 (t, J = 5.6, 6H, CH_3); MS (HR-ESI) *m/z* $[M + H]^+$ found 461.1442$(M+H)^+$

4.2.15. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl (methyl-*L*-leucyl)sulfamate (31)

^{1}H NMR (500 MHz, CD_3OD): δ 8.51 (s, 1H, Ar, 8H), 8.19 (s, 1H, Ar, 2H), 6.08 (d, J = 5.70, 1H, CH, 1'H), 4.65 (t, J = 5.3, 1H, CH, 2'H), 4.39 (m, 1H, CH, 3'H), 4.32 (m, 2H, CH_2, 5'H), 4.28 (m, 1H, CH2, 4'H), 3.12 (t, J = 7.7, 1H, CH), 2.39 (s, 3H, CH_3), 1.73 (m, 1H, CH), 1.56 (m, 1H, CH), 1.38 (m, 1H, CH), 0.90 (dd, J = 27.9, 6.1, 6H, CH_3); MS (HR-ESI) *m/z* $[M + H]^+$ found 474.1773.

4.2.16. ((2*R*,3*S*,5*R*)-5-(6-amino-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methyl ((2*R*,3*S*)-2,3-dihydroxy-4-methylpentanoyl)sulfamate (34)

^{1}H NMR (500 MHz, CD_3OD): δ 8.51 (s, H_8), 8.18 (s, H_2), 6.49 (t, J = 6.85 Hz, $H_{1'}$), 4.63-4.62 (m, $H_{4'}$), 4.30-4.24 (m, $H_{3'}$, H_α), 4.19 (d, J = 3.70 Hz, $H_{5'}$), 4.06 (d-like, $H_{5''}$), 3.5 (dd, J = 8.85, 1.25 Hz, H_β), 2.75 (qd, J = 7.25, 3.60 Hz, $H_{2'}$), 2.46 (qd, J = 6.05, 3.10 Hz, $H_{2''}$), 1.88-1.81 (m, Hγ), 0.99 (d, J = 6.70 Hz, 3H), 0.94 (d, J = 6.70 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 182.2, 158.0, 154.5, 151.1, 142.1, 120.8, 87.55, 86.3, 80.6, 75.4, 73.7, 70.1, 42.4, 32.9, 20.6, 20.4;MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{24}N_6O_8S$: 461.1454; found: 461.1408.

4.2.17. ((2*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-4-hydroxytetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl)sulfamate (36)

^{1}H NMR (300 MHz, CD_3OD): δ 8.45 (s, H_8), 8.18 (s, H_2), 5.99 (d, J = 1.83 Hz, $H_{1'}$), 4.71-4.65 (m, $H_{4'}$, $H_{2'}$), 4.44 (dd, J = 11.34, 2.55 Hz, $H_{5'}$), 4.27 (dd, J = 11.19, 3.30 Hz, $H_{5''}$), 4.08 (dd, J = 9.15, 3.66 Hz, H_α), 2.41 (qd, J = 8.97, 2.49 Hz, $H_{3'}$), 2.11 (qd, J = 6.03, 2.88 Hz, $H_{3''}$), 1.82-1.80 (m, H_β), 1.60-1.42 (m, $H_{\beta''}$, H_γ), 0.91 (d, J = 2.58 Hz, 3H), 0.89 (d, J

= 2.73 Hz, 3H).

4.2.18. ((2*R*,3*S*,5*R*)-5-(6-amino-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methyl ((*S*)-2-hydroxy-4-methylpentanoyl)sulfamate (37)

^{1}H NMR (600 MHz, CD_3OD): δ 8.47 (s, H_8), 8.18 (s, H_2), 6.49 (dd, J = 7.32, 5.94 Hz, $H_{1'}$), 4.62 (quintet, J = 2.76Hz, $H_{4'}$), 4.28-4.23 (m, $H_{5'}$, $H_{5''}$), 4.20 (q, J = 3.66 Hz, $H_{3'}$), 3.99 (dd, J = 9.60, 3.18 Hz, H_α), 2.77 (qd, J = 7.80, 6.00 Hz, $H_{2'}$), 2.46 (qd, J = 5.94, 0.60 Hz, $H_{2''}$), 1.86-1.80 (m, Hγ), 1.57 (qd, J = 9.18, 3.66 Hz, H_β), 1.45 (qd, J = 9.60, 5.04 Hz, H_β), 0.92 (d, J = 6.36 Hz, 3H), 0.90 (d, J = 6.90 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 184.1, 158.0, 154.6, 151.1, 142.0, 120.9, 87.5, 86.5, 73.8, 70.2, 46.4, 42.3 (2C), 26.5, 24.8, 22.7; MS (HR-ESI) *m/z* $[M + H]^+$ calc. for $C_{16}H_{24}N_6O_7S$: 445.1505; found: 445.1473.

4.2.19. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-3-hydroxy-2-methoxy-4-methylpentanoyl)sulfamate (38)

^{1}H NMR (300 MHz, CD_3OD): δ 8.46 (s, H_8), 8.19 (s, H_2), 6.08 (d, J = 5.31 Hz, $H_{1'}$), 4.66 (t, J = 5.49 Hz, $H_{2'}$), 4.40-4.28 (m, $H_{3'}$, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.70 (d, J = 3.48 Hz, H_α), 3.42-3.41 (m, H_β), 3.33 (s, 3H), 1.88-1.81 (m, H_γ), 0.98 (d, J = 6.78 Hz, 3H), 0.91 (d, J = 6.78 Hz, 3H).

4.2.20. ((2*R*,3*S*,5*R*)-5-(6-amino-9H-purin-9-yl)-3-hydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dimethoxy-4-methylpentanoyl)sulfamate (42)

^{1}H NMR (300 MHz, CD_3OD): δ 8.36 (s, 1H), 8.14 (s, 1H), 6.39 (t, J = 6.60 Hz, 1H), 4.55 (quintet, J = 2.94 Hz, 1H), 4.39-4.28 (m, 2H), 4.13 (q, J = 3.27 Hz, 1H), 3.63 (d, J = 3.12 Hz, 1H), 3.28 (s, 3H), 3.21 (s, 3H), 3.10 (dd, J = 7.68, 3.87 Hz, 1H), 2.70 (quintet, J = 7.14 Hz, 1H), 2.43-2.32 (m, 1H), 1.89-1.77 (m, 1H), 0.87 (d, J = 6.60 Hz, 3H), 0.80 (d, J = 6.78 Hz, 3H).

4.2.21. ((2*R*,3*S*,4*R*,5*R*)-5-(6-amino-9H-purin-9-yl)-3,4-dihydroxytetrahydrofuran-2-yl)methyl ((2*S*,3*R*)-2,3-dimethoxy-4-methylpentanoyl)sulfamate (43)

^{1}H NMR (300 MHz, CD_3OD): δ 8.42 (s, H_8), 8.28 (s, H_2), 6.07 (d, J = 4.95 Hz, $H_{1'}$), 4.67 (t, J = 4.68 Hz, $H_{4'}$), 4.61-4.50 (m, $H_{5'}$, $H_{5''}$), 4.41 (t, J = 5.85 Hz, $H_{3'}$), 4.31 (t, J = 3.48 Hz, $H_{2'}$), 3.71 (d, J = 3.66 Hz, H_α), 3.36 (s, 3H), 3.33 (s, 3H), 3.17-3.11 (m, H_β), 2.01 (hextet, J = 6.75 Hz, H_γ), 0.99 (d, J = 6.60 Hz, 3H), 0.89 (d, J = 6.51 Hz, 3H).

4.2.22. ((3a*R*,4*S*,6*R*,6a*R*)-6-(6-amino-9H-purin-9-yl)-2-iminohexahydrofuro[3,4-d]oxazol-4-yl)methyl ((2*S*,3*R*)-2,3-dihydroxy-4-methylpentanoyl)sulfamate (44)

^{1}H NMR (500 MHz, CD_3OD): δ 8.71 (s, 1H, Ar, 8H), 8.58 (s, 1H, Ar, 2H), 6.34 (s, 1H, CH, 1'H), 4.47 (t, 1H, CH, 2'H), 4.43 (d, 1H, CH, 3'H), 4.28 (d, 1H, CH, 4'H), 4.23 (m, 1H, CH_2, 5'H), 4.07 (s, 1H, CH), 3.48 (s, 1H, CH), 1.85 (m, 1H, CH), 0.98 (dd, 6H, CH_3); MS (FAB) *m/z* 502 (M+H).

IV. Part 3. Discovery of Simplified Leucyladenylate Sulfamates

as a Novel LRS-targeted mTORC1 Inhibitors

1. Design background & strategy

In our continuing effort to develop LRS-targeted mTORC1 inhibitors as a potential anti-cancer agent, we decided to design new scaffolds based on the structures of leucyladenylate sulfamate analogues from our previous study.[43] Although leucyladenylate sulfamate analogues demonstrated highly potent and selective cytotoxicity against human colon cancer cells, high polarity of these compounds may hinder further pre-clinical development due to synthetic difficulties and poor bioavailability. We devised a simplified scaffold by introducing a benzenesulfonamide group that replaces 5-*O*-sulfamoylribose as shown in **Figure 27**. In this new scaffold, we also replaced the adenine group with 3,4-dimethoxyphenyl (**2a**) or 2-phenoxy (**2b**) groups that were chosen based on a simple immunoblot-based screening of a commercial chemical library with approximately 400 compounds. Additionally, we synthesized constrained analogues by introducing a cyclic urea group that connects R^2 and the amide nitrogen, based on our previous finding that a leucinol analogue that contained a cyclic urea, (*S*)-4-isobutyloxazolidin-2-one, demonstrated the most potent inhibition of mTORC1.[25]

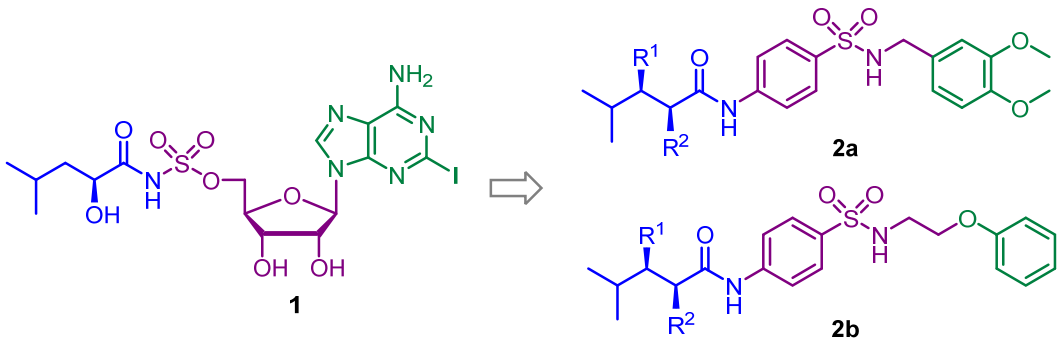
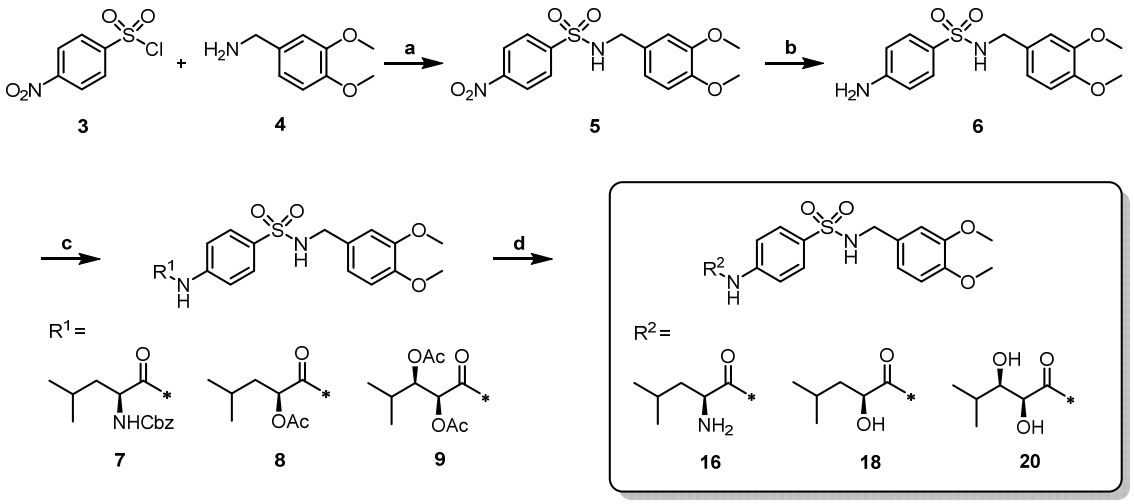


Figure 27. Leucyladenylate sulfamate (**1**) and its simplified surrogates.

2. Result and Discussion

2.1. Chemistry

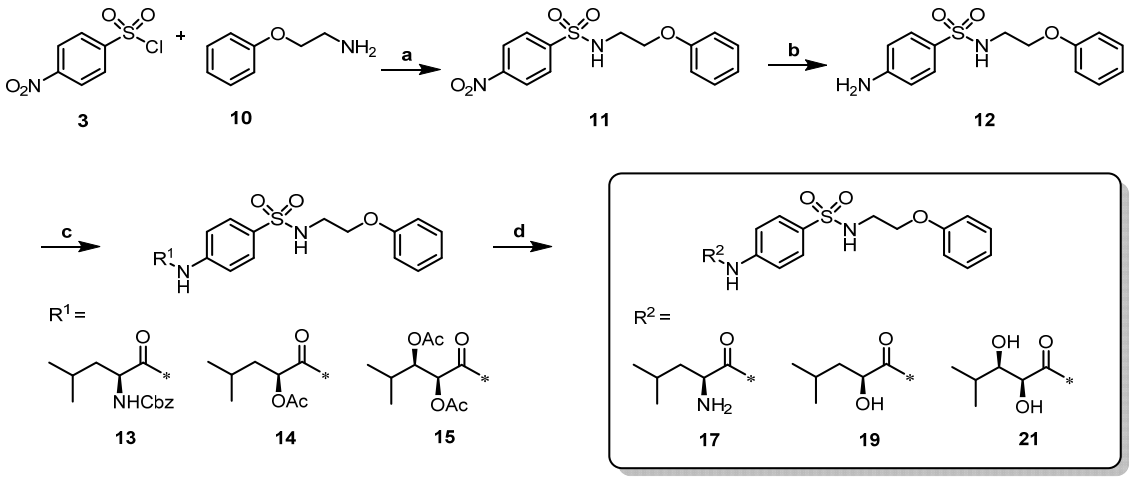
3,4-Dimethoxybenzyl analogues (**16**, **18**, **20**) were synthesized by following the pathway described in **Scheme 20**. Commercially available 4-nitrobenzenesulfonyl chloride (**3**) and 3,4-dimethoxybenzylamine (**4**) were reacted to give the sulfonamide **5**, which was subsequently reduced to afford the aniline intermediate **6**. Compound **6** was then condensed with *N*-Cbz leucine, *O*-diacetyl protected (2*S*,3*R*)-dihydroxy-4-methylpentanoic acid (DMPA) or *O*-acetyl protected (2*S*)-hydroxyisocaproic acid (HICA, *L*-leucic acid) to yield compounds **7-9**, respectively. The acetyl protected DMPA and HICA used for these reactions were prepared from commercially available 4-methyl-2-pentenoic acid and *L*-leucic acid respectively, by following previously reported procedures.[36] Deprotection of the α-amino or α-hydroxyl group of compounds **7-9** provided the final compounds **16, 18,** and **20**.



Scheme 20. Synthesis of 3,4-dimethoxybenzyl analogues.

Reagents & conditions: (a) TEA, MC, 0 ºC to r.t, 2 h; (b) Pd/C, H_2, MeOH, r.t, 2 h; (c) i) *N*-Cbz leucine, cyanuric chloride, TEA, acetone for **7**, $(COCl)_2$, anhyd. DMF, MC, *O*-diacetyl protected chiral acid for **8** and *O*-acetyl protected *L*-leucic acid for **9**, 0 ºC to r.t, 2 h, ii) **6**, DIPEA, anhyd. DMF, 0 ºC to r.t, 2 h; (d) Pd/C, H_2, 2N NH_3 in MeOH, r.t, overnight for **16**, 0.02M NaOMe, r.t, 2 h for **18** and **20**.

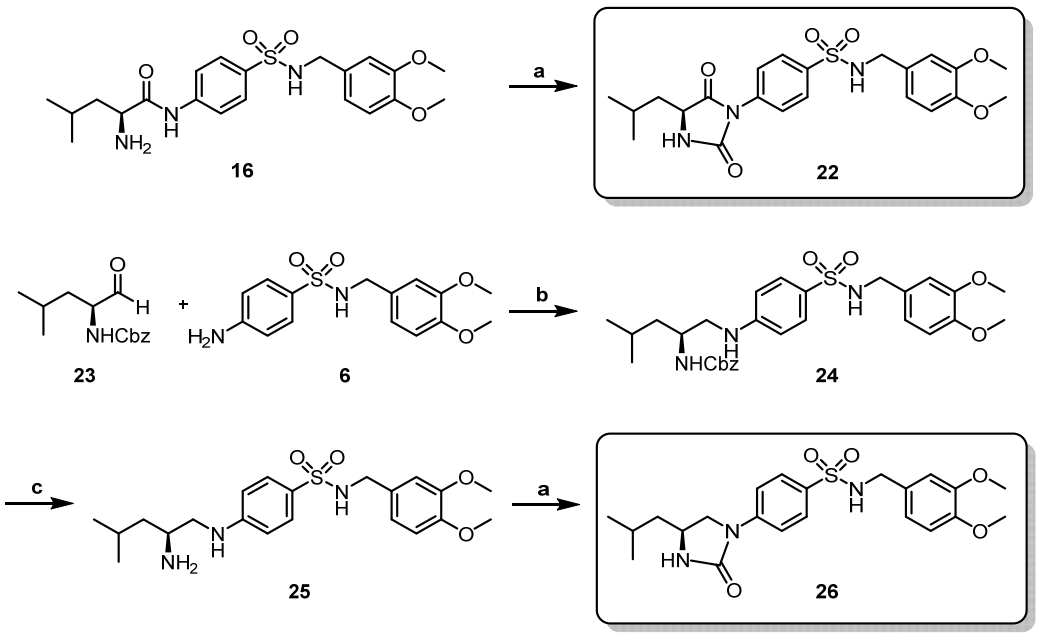
Syntheses of phenoxyethyl analogues (**17**, **19**, **21**) began with coupling commercially available 4-nitrobenzenesulfonyl chloride (**3**) and 2-phenoxyethylamine (**10**) as shown in **Scheme 21**. Compounds **11-15, 17, 19,** and **21** were synthesized by following the same procedures described in **Scheme 20**.



Scheme 21. Synthesis of phenoxyethyl analogues.

Reagents & conditions: (a) TEA, MC, 0 ºC to r.t, 2 h; (b) Pd/C, H_2, MeOH, r.t, 2 h; (c) i) *N*-Cbz leucine, cyanuric chloride, TEA, acetone for **13**, $(COCl)_2$, anhyd. DMF, MC, *O*-diacetyl protected chiral acid for **14** and *O*-acetyl protected *L*-leucic acid for **15**, 0 ºC to r.t, 2 h, ii) **12**, DIPEA, anhyd. DMF, 0 ºC to r.t, 2 h; (d) Pd/C, H_2, 2N NH_3 in MeOH, r.t, overnight for **17**, 0.02M NaOMe, r.t, 2 h for **19** and **21**.

Cyclic urea analogues of **16** (**22** and **26**) were synthesized as shown in **Scheme 22**. Carbonylation of **16** by using 1,1'-carbonyldiimidazole (CDI) provided the cyclic urea **22**. Reductive amination of *N*-Cbz leucinal (**23**) prepared from *N*-Cbz leucine, with the amine **6** gave compound **24**. Subsequent deprotection reaction followed by carbonylation with CDI yielded the desired compound **26**.



Scheme 22. Synthesis of imidazolidin-2-one analogues.

Reagents & conditions: (a) 1,1'-carbonyldiimidazole, DMF, r.t, 12 h; (b) $NaBH_3CN$, AcOH, MeOH, r.t, 12 h; (c) Pd/C, H_2, 2N NH_3 in MeOH, r.t, overnight.

2.2. Biological Activity

To evaluate the effect of the synthesized compounds on cellular mTORC1 pathway, we first determined leucine-induced phosphorylation of S6Kinase (S6K) in HEK293 cells by immunoblots. In our previous studies, we have demonstrated that LRS-targeted inhibitors block the phosphorylation of S6K by inhibiting the mTORC1 pathway.[25,30] For a primary screening, we pretreated HEK293 cells with compounds **16-21** at one fixed concentration (200 μM) as well as with compound **1** and rapamycin (100 nM) for comparison, and then activated mTORC1 by treating the cells with leucine for 10 min. As shown in **Figure 28a**, leucine induced phosphorylation of S6K, demonstrating an intense band that corresponds to pS6K whereas rapamycin and compound **1** inhibited S6K phosphorylation. Among the

tested compounds, **16** appeared to inhibit S6K phosphorylation to a similar extent as compound **1**. Based on band intensities of pS6K, compounds **18, 20**, and **21** appeared to inhibit S6K phosphorylation slightly, while compounds **17** and **19** did not affect phosphorylation, suggesting that 3,4-dimethoxybenzyl analogues (**16**, **18**, **20**) were more potent than the corresponding 2-phenoxyethyl analogues (**17**, **19**, **21**). In contrast to our previous observation that leucyladenylate sulfamate with an α-hydroxyl group (compound **1**) demonstrated better inhibition than R^2-amino or R^1, R^2-dihydroxyl surrogates,[30] compounds with an α-amino group (**16** and **17**) showed more potent inhibition than the α-hydroxyl group containing counter parts (**18** and **19**), suggesting that removing the adenylate may alter the binding interactions. When we examined expression levels of S6K, LRS and mTOR, these levels were not affected by the treatment of each compound.

Next, to determine dose-dependent inhibition of S6K phosphorylation, we decided to focus on the most potent analogue, compound **16** (**Figure 28b)**. Compound **16** inhibited S6K phosphorylation in a dose-dependent manner, showing a significant reduction in band intensities starting at 50 μM. Compared to our previous studies requiring concentrations greater than 100 μM of compounds to observe a similar degree of inhibition, compound **16** appeared to be the most potent among the compounds we have developed by far.

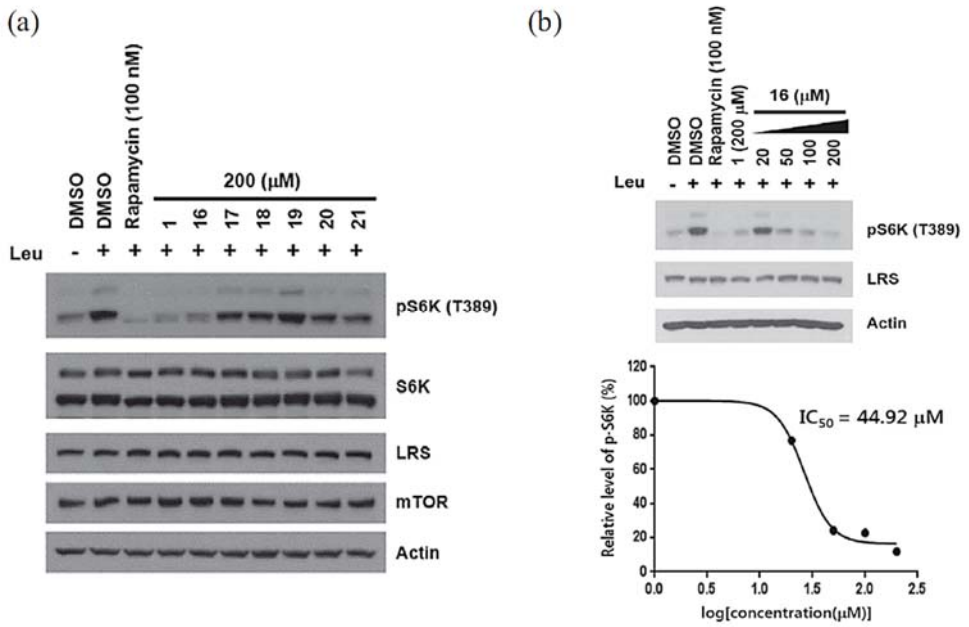


Figure 28. (a) Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds at fixed concentrations; (b) Dose-dependent inhibition of mTORC1 by compound **16**.

Next, we evaluated the inhibitory effect of two constrained analogues, **22** and **26**, which shared the basic scaffold of compound **16**, but were further modified based on the structure of (*S*)-4-isobutyloxazolidin-2-one, a cyclic derivative of leucinol.[25] To our surprise, compound **22** inhibited S6K phosphorylation dose-dependently, even more potent than compound **16**; however, compound **26** completely lost the inhibitory effect (**Figure 29**). While more detailed SAR studies are needed to explain such a big activity difference between **22** and **26**, we speculated that the introduction of the conformational constraint probably enhanced the inhibitory effect of compound **22**. On the contrary, the removal of a leucine carbonyl group adversely affected binding interaction of compound **26**, possibly leading to the loss of critical binding interactions with the leucine recognition

site of LRS.

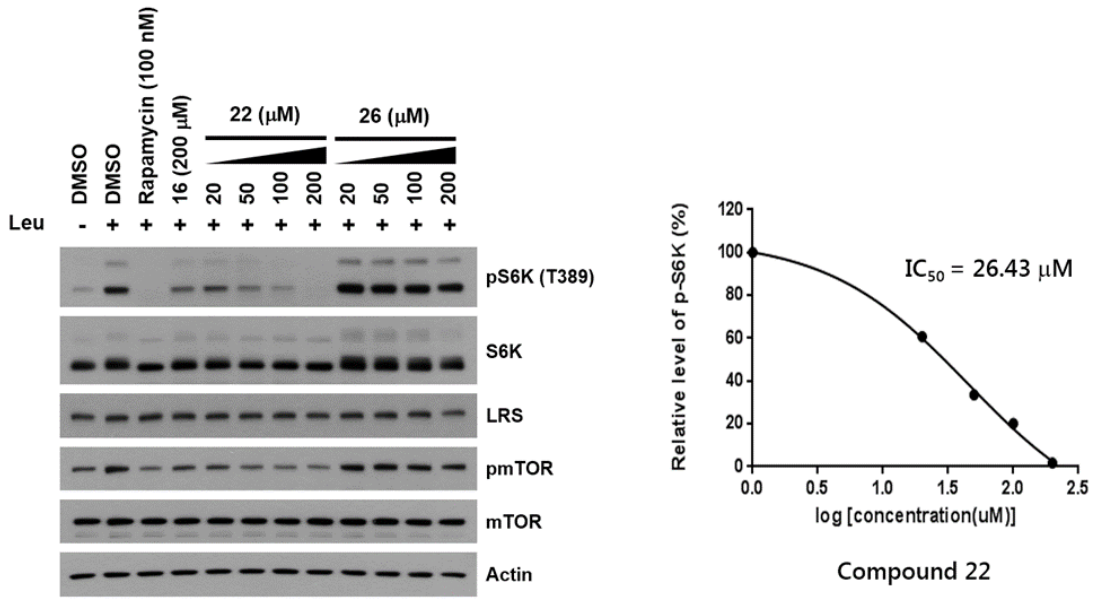


Figure 29. Dose-dependent inhibition of leucine-induced mTORC1 activation of compounds **22** and **26** in HEK293 cells.

To examine whether the inhibitory mechanism of these simplified analogues is related to the catalytic activity of LRS, we performed aminoleucylation assays with compounds **16** and **22**. As shown in **Figure 30**, both compounds showed poor LRS inhibition with the IC_{50} values greater than 800 μM, proving that these compounds only acted upon the LRS-mediated mTORC1 pathway without affecting the canonical function of LRS. Given that previously developed leucyladenylate sulfamates have the IC_{50} values ranging from 22 nM to 337 nM,[30] this result indicates that the replacement of the adenylate group with a 3,4-dimethoxybenzyl group not only enhanced the inhibitory effect for mTORC1, but also improved selectivity.

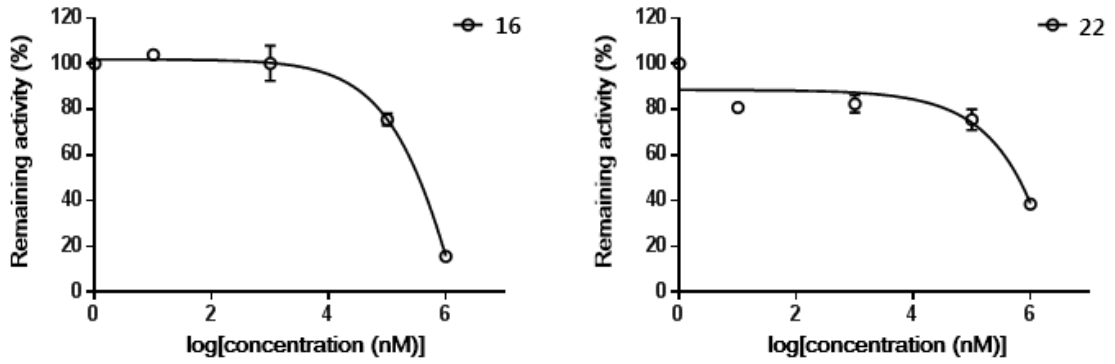


Figure 30. Inhibition of catalytic leucylation by compounds **16** and **22**.

Finally, to evaluate the anticancer activity of **16** and **22**, we carried out the sulforhodamine B (SRB) colorimetric assays for cytotoxicity.[38] A total of six different types of cancer cell lines were treated with compounds **16** and **22**, in parallel with etoposide as a positive control. To determine cancer cell-specific cytotoxicity, normal human lung epithelial cells (MRC-5) were also treated with each compound. As shown in **Table 2**, both compounds demonstrated similar IC_{50} values for all six types of cancer cells while exhibited much higher IC_{50} values for normal MRC-5 cells. Although we did not observe colon cancer-specific cytotoxicity as demonstrated with compound **1** in our previous study, both compounds **16** and **22** showed general cytotoxicity towards various cancer cells without exerting significant toxicity on normal cells. Compounds **16** and **22** also appeared to be slightly less cytotoxic than etoposide, which is likely due to their improved selectivity toward mTORC1.

Table 2. Relative cell growth inhibition of compounds **16** and **22** for various cancer cell lines and normal cells. [a]

IC_{50} (μM)	A549	HCT116	K562	MDA-MB-231	SK-HEP-1	SNU638	MRC5
16	5.29	3.96	4.48	5.44	3.07	6.26	> 20
22	5.54	4.28	2.86	5.65	2.44	5.22	> 20
Etoposide	0.30	1.06	0.76	1.53	0.63	1.05	11.73

[a]A549, lung cancer cells; HCT116, colon cancer cells; K562, leukemia cells; MDA-MB-231, breast cancer cells; SK-Hep-1, liver cancer cells; SNU638, stomach cancer cells; MRC5, lung normal epithelial cell.

3. Conclusion

We have developed a new series of LRS-targeted mTORC1 inhibitor based on the structures of previously identified inhibitors. In this new series, we replaced the adenylate group of leucyladenylate sulfamate (**1**) with a *N*-(3,4-dimethoxybenzyl)benzenesulfonamide group or a *N*-(2-phenoxyethyl)benzenesulfonamide group that can maintain specific binding, but has more favorable physicochemical properties such as reduced polarity and asymmetric centers. 3,4-Dimethoxybenzyl analogues generally showed better inhibition than 2-phenoxyethyl analogues; additionally, within each series, the presence of an α-amino group in the R^2 position appears to be important for inhibition of the mTORC1 pathway. In particular, compound **16** and its constrained analogue **22** effectively inhibited S6K phosphorylation in a dose-dependent manner without affecting catalytic leucylation activity of LRS. Furthermore, these compounds exhibited cancer cell specific cytotoxicity against six different types of cancer cell lines, again supporting that the LRS-mediated

mTORC1 pathway is a viable target for novel anticancer therapy. Currently, we are expanding our library of simplified analogues for in-depth SAR studies to improve selectivity and cytotoxicity of LRS-targeted mTORC1 inhibitors.

4. Experimental

4.1. General Experimental

All chemical reagents were commercially available. Silica gel column chromatography was performed on Silica Gel 60, 230–400 mesh, Merck. Proton NMR spectra were recorded on a JEOL JNM-LA 300 at 300 MHz and Bruker Analytik, DE/AVANCE Digital 400 at 400 MHz or Bruker AMX-500 (500 MHz) spectrometer. Chemical shifts are reported in ppm units with tetramethylsilane as a reference standard. Mass spectra and HRMS results were recorded on VG Trio-2 GC－MS instrument and JEOL JMS-AX instrument, respectively.

4.2. General procedure

4.2.1. Sulfonamide synthesis (Procedure A).

A solution of 3,4-dimethoxybenzylamine **4** or 2-phenoxyethylamine **10** (1.2 equiv.) in MC was cooled to 0^{o}C and triethylamine (2.4 equiv.) was added. The resulting solution was stirred for 10 min and 4-nitrobenzenesulfonyl chloride **3** (1 equiv.) was added to the mixture and then stirred for 2 h at room temperature. The reaction was quenched with water and extracted with EtOAc twice. The combined organic extracts were dried over

$MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding sulfonamide product.

4.2.2. Nitro reduction (Procedure B).

A solution of 4-nitrobenzensulfonamide derivatives in methanol was added 10% Pd/C catalyst (0.1 mg/1 mg) and stirred under hydrogen gas in balloon for 2 h at room temperature. After completed, the reaction mixture was filtered on celite pad, evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding aniline product.

4.2.3. Amide coupling (Procedure C).

Method A: a solution of *N*-Cbz leucine (3 equiv.) in acetone was cooled to 0°C and cyanuric chloride (3 equiv.) followed by triethylamine (6 equiv.) was added. The reaction mixture was stirred for 2 h at room temperature. After *in situ* generation of acyl chloride completed, the solvent was evaporated and the mixture was dissolved in DMF. Aniline (1 equiv.) and DIPEA (3 equiv.) were added at 0°C and the reaction mixture was stirred for 2 h at room temperature. The reaction was quenched with aqueous NH_4Cl and extracted with EtOAc twice. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding amide product.

Method B: a solution of carboxylic acid (DMPA or HICA, 3 equiv.) in anhydrous MC was cooled to 0°C and oxalyl chloride (3.6 equiv.) followed by anhyd. DMF (cat.) was

added. The reaction mixture was stirred for 2 h at room temperature. After *in situ* generation of acyl chloride completed, the solvent was evaporated and the mixture was dissolved in DMF. Aniline (1 equiv.) and DIPEA (3 equiv.) were added at 0^{o}C and the reaction mixture was stirred for 2 h at room temperature. The reaction was quenched with aqueous NH_4Cl and extracted with EtOAc twice. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding amide product.

4.2.4. Cbz deprotection (Procedure D).

A solution of starting material in 2N NH_3 in methanol was added 10% Pd/C catalyst (0.1 mg/1 mg) and stirred under hydrogen gas in balloon for overnight at room temperature. After completed, the reaction mixture was filtered on celite pad, evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding final compound.

4.2.5. Acetyl deprotection (Procedure E).

The starting material was dissolved in 0.02 M sodium methoxide solution in methanol (0.1 ml/1 mg) and stirred for 2 h at room temperature. DOWEX 50WX8 hydrogen form resin was added in portions to adjust the pH 7, filtered and concentrated to afford the corresponding final compound.

4.2.6. CDI coupling (Procedure F).

A solution of diamine (1 equiv.) in DMF was added 1,1'-carbonyldiimidazole (1.1 equiv.)

at room temperature and stirred for 12 h. The reaction was quenched with water and extracted with EtOAc twice. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding cyclic urea product.

4.2.7. Reductive amination (Procedure G).

To a mixture of aldehyde (1 equiv.) and aniline (1 equiv.) in methanol was added acetic acid (1 equiv.) followed by $NaBH_3CN$ (5 equiv.) and the reaction mixture was stirred for 12 h at room temperature. The reaction was quenched with aqueous $NaHCO_3$ and extracted with EtOAc twice. The combined organic extracts were dried over $MgSO_4$, filtered, and evaporated in vacuo. The residue was purified by column chromatography over silica gel to afford the corresponding product.

4.3. Chemical spectra

4.3.1. *N*-(3,4-Dimethoxybenzyl)-4-nitrobenzenesulfonamide (5).

Compound **5** was synthesized by following the general procedure A. Yield 90%, white solid; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 8.31 (d, 2H, J = 8.27 Hz), 7.99 (d, 2H, J = 8.27 Hz), 6.64-6.72 (m, 3H), 4.91 (m, 1H), 4.13 (m, 2H), 3.81 (s, 3H), 3.79 (s, 3H); MS (ESI) *m/z* 353 $[M+H]^+$.

4.3.2. 4-Amino-*N*-(3,4-dimethoxybenzyl)benzenesulfonamide (6).

Compound **6** was synthesized by following the general procedure B. Yield 97%, yellow

solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.50 (m, 2H), 6.63-6.82 (m, 5H), 3.93 (s, 2H), 3.77 (s, 3H), 3.74 (s, 3H); MS (ESI) *m/z* 323 $[M+H]^+$.

4.3.3. Benzyl (*S*)-(1-((4-(N-(3,4-dimethoxybenzyl)sulfamoyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (7).

Compound **7** was synthesized by following the general procedure C (method A). Yield 76%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.62 (s, 4H), 7.21-7.26 (m, 5H), 6.61-6.70 (m, 3H), 5.00 (s, 2H), 4.19 (m, 1H), 3.91 (s, 2H), 3.65 (s, 3H), 3.61 (s, 3H), 1.62 (m, 1H), 1.53 (m, 2H), 0.89 (m, 6H); MS (ESI) *m/z* 570 $[M+H]^+$.

4.3.4. (*S*)-1-((4-(*N*-(3,4-Dimethoxybenzyl)sulfamoyl)phenyl)amino)-4-methyl-1-oxopentan-2-yl acetate (8).

Compound **8** was synthesized by following the general procedure C (method B). Yield 62%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.72 (d, 2H, *J* = 8.61 Hz), 7.59 (d, 2H, *J* = 8.79 Hz), 6.67 (m, 3H), 4.71 (t, 1H, *J* = 6.03 Hz), 3.98 (t, 1H, *J* = 6.03 Hz), 3.77 (s, 3H), 3.74 (s, 3H), 2.16 (s, 3H), 1.79 (m, 1H), 1.70 (m, 2H), 0.90 (t, 1H, *J* = 6.03 Hz); MS (ESI) *m/z* 479 $[M+H]^+$.

4.3.5. (2*S*,3*R*)-1-((4-(*N*-(3,4-Dimethoxybenzyl)sulfamoyl)phenyl)amino)-4-methyl-1-oxopentane-2,3-diyl diacetate (9).

Compound **9** was synthesized by following the general procedure C (method B). Yield 55%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.73 (d, 2H, *J* = 8.97 Hz), 7.65 (d, 2H, *J* = 8.97 Hz), 6.69-6.80 (m, 3H), 5.27 (d, 1H, *J* = 2.86 Hz), 5.17 (dd, 1H, *J* = 8.8, 3.1 Hz), 4.01 (s, 2H), 3.76 (s, 3H), 3.70 (s, 3H), 2.20 (s, 3H), 2.03 (s, 3H), 2.03 (m, 1H), 0.98

(dd, 6H, *J* = 9.72, 6.78 Hz); MS (FAB) *m/z* 559 $[M+Na]^+$.

4.3.6. 4-Nitro-*N*-(2-phenoxyethyl)benzenesulfonamide (11).

Compound **11** was synthesized by following the general procedure A. Yield 96%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.25 (d, 2H, *J* = 8.97 Hz), 7.99 (d, 2H, *J* = 8.97 Hz), 7.18 (t, 3H, *J* = 7.32 Hz), 6.90 (d, 1H, *J* = 7.53 Hz), 6.69 (d, 2H, *J* = 7.68 Hz), 5.10 (t, 1H, *J* = 6.03 Hz), 3.93 (t, 2H, *J* = 4.77 Hz), 3.39 (q, 2H, *J* = 5.49 Hz); MS (ESI) *m/z* 323 $[M+H]^+$.

4.3.7. 4-Amino-*N*-(2-phenoxyethyl)benzenesulfonamide (12).

Compound **12** was synthesized by following the general procedure B. Yield 89%, yellow solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.41-7.47 (m, 2H), 7.09-7.16 (m, 2H), 6.73-6.83 (m, 3H), 6.54-6.61 (m, 2H), 3.84 (t, 2H, *J* = 5.71 Hz), 3.09 (t, 2H, *J* = 5.71 Hz); MS (ESI) *m/z* 293 $[M+H]^+$.

4.3.8. Benzyl (*S*)-(4-methyl-1-oxo-1-((4-(*N*-(2-phenoxyethyl)sulfamoyl)phenyl)amino)pentan-2-yl)carbamate (13).

Compound **13** was synthesized by following the general procedure C (method A). Yield 87%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.67-7.81 (m, 4H), 7.27.-7.37 (m, 4H), 7.16-7.23 (m, 3H), 6.89 (m, 1H), 6.78 (d, 2H, *J* = 7.89 Hz), 5.09 (s, 2H), 4.31 (m, 1H), 3.92 (t, 2H, *J* = 5.5 Hz), 3.26 (t, 2H, *J* = 5.55 Hz), 1.53-1.74 (m, 3H), 0.97 (dd, 6H, *J* = 6.47, 2.62 Hz); MS (FAB) *m/z* 562 $[M+H]^+$.

4.3.9. (*S*)-4-Methyl-1-oxo-1-((4-(*N*-(2-phenoxyethyl)sulfamoyl)phenyl)amino)pentan-2-yl acetate (14).

Compound **14** was synthesized by following the general procedure C (method B). Yield 65%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.72 (d, 2H, *J* = 8.97 Hz), 7.64 (d, 2H, *J* = 8.97 Hz), 7.10 (t, 2H, *J* = 7.50 Hz), 6.78 (t, 2H, *J* = 7.50 Hz), 6.68 (d, 1H, *J* = 8.79 Hz), 4.97 (dd, 1H, *J* = 9.51, 3.84 Hz), 3.82 (t, 2H, *J* = 5.49 Hz), 3.17 (t, 2H, *J* = 5.49 Hz), 2.05 (s, 3H), 1.72 (m, 2H), 1.53 (m, 1H), 0.89 (dd, 6H, *J* = 6.42, 4.95 Hz); MS (ESI) *m/z* 449 $[M+H]^+$.

4.3.10. (2*S*,3*R*)-4-Methyl-1-oxo-1-((4-(N-(2-phenoxyethyl)sulfamoyl)phenyl)amino)pentane-2,3-diyl diacetate (15).

Compound **15** was synthesized by following the general procedure C (method B). Yield 72%, colorless oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.81 (d, 2H, *J* = 8.79 Hz), 7.67 (d, 2H, *J* = 8.79 Hz), 7.21 (t, 2H, *J* = 8.61 Hz), 6.88 (t, 2H, *J* = 7.32 Hz), 6.79 (d, 1H, *J* = 7.68 Hz), 5.28 (d, 1H, *J* = 2.94 Hz), 5.17 (dd, 1H, *J* = 8.79, 2.76 Hz), 3.93 (t, 2H, *J* = 5.52 Hz), 3.26 (t, 2H, *J* = 5.49 Hz), 2.06 (m, 1H), 0.98 (dd, 6H, *J* = 9.9, 6.78 Hz); MS (ESI) *m/z* 507 $[M+H]^+$.

4.3.11. (*S*)-2-Amino-*N*-(4-(*N*-(3,4-dimethoxybenzyl)sulfamoyl)phenyl)-4-methylpentanamide (16).

Compound **16** was synthesized by following the general procedure D. Yield 88%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.64 (m, 4H), 6.66 (m, 3H), 3.92 (s, 2H), 3.66 (s, 3H), 3.62 (s, 3H), 3.60 (m, 1H), 1.44-1.70 (m, 3H), 0.90 (dd, 6H, *J* = 6.39, 3.66 Hz);

HRMS (FAB) calcd for $C_{21}H_{30}N_3O_5S^+$ $[M+H]^+$: 436.1828, found: 436.1861.

4.3.12. (*S*)-2-Amino-4-methyl-*N*-(4-(*N*-(2-phenoxyethyl)sulfamoyl)phenyl)pentanamide (17).

Compound **17** was synthesized by following the general procedure D. Yield 94%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.70 (m, 4H), 7.11 (m, 2H), 6.79 (t, 1H, *J* = 7.50 Hz), 6.70 (m, 2H), 3.83 (t, 2H, *J* = 5.49 Hz), 3.39 (dd, 1H, *J* = 8.20, 6.03 Hz), 3.16 (t, 2H, *J* = 5.50 Hz), 1.33-1.72 (m, 3H), 0.88 (t, 6H, *J* = 5.50 Hz); HRMS (FAB) calcd for $C_{20}H_{28}N_3O_4S^+$ $[M+H]^+$: 406.1722, found: 406.1776.

4.3.13. (*S*)-*N*-(4-(*N*-(3,4-Dimethoxybenzyl)sulfamoyl)phenyl)-2-hydroxy-4-methylpentanamide (18).

Compound **18** was synthesized by following the general procedure E. Yield 85%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.69 (m, 4H), 6.68 (m, 3H), 4.09 (t, 1H, *J* = 6.78 Hz), 3.91 (s, 2H), 3.67 (s, 3H), 3.62 (s, 3H), 1.83 (m, 1H), 1.51 (m, 2H), 0.90 (d, 3H, *J* = 2.19 Hz), 0.88 (d, 3H, *J* = 2.19 Hz); MS (ESI) calcd for $C_{21}H_{29}N_2O_6S^+$ $[M+H]^+$: 437.1668, found: 437.1752.

4.3.14. (*S*)-2-Hydroxy-4-methyl-*N*-(4-(*N*-(2-phenoxyethyl)sulfamoyl)phenyl)pentanamide (19).

Compound **19** was synthesized by following the general procedure E. Yield 88%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.71 (m, 4H), 7.11 (t, 2H, *J* = 8.58 Hz), 6.79 (t, 2H, *J* = 7.50 Hz), 6.71 (t, 2H, *J* = 8.61 Hz), 4.09 (t, 1H, *J* = 7.14 Hz), 3.84 (t, 2H, *J* = 5.52

Hz), 3.16 (t, 2H, *J* = 5.52 Hz), 1.80 (m, 1H), 1.51 (m, 2H), 0.89 (dd, 6H, *J* = 6.60, 2.19 Hz); HRMS (FAB) calcd for $C_{20}H_{27}N_2O_5S^+$ $[M+H]^+$: 407.1562, found: 407.1602.

4.3.15. (2*S*,3*R*)-*N*-(4-(*N*-(3,4-Dimethoxybenzyl)sulfamoyl)phenyl)-2,3-dihydroxy-4-methylpentanamide (20).

Compound **20** was synthesized by following the general procedure E. Yield 80%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.77 (m, 4H), 6.78 (s, 1H), 6.72 (m, 2H), 4.25 (d, 1H, *J* = 1.81 Hz), 4.00 (s, 2H), 3.76 (s, 3H), 2,92 (s, 3H), 3.52 (dd, 1H, *J* = 8.97, 1.83 Hz), 1.89-2.01 (m, 1H), 1.02 (dd, 6H, *J* = 6.80, 3.40 Hz); HRMS (FAB) calcd for $C_{21}H_{29}N_2O_7S^+$ $[M+H]^+$: 453.1617, found: 453.1657.

4.3.16. (2*S*,3*R*)-2,3-Dihydroxy-4-methyl-*N*-(4-(*N*-(2-phenoxyethyl)sulfamoyl)phenyl)pentanamide (21).

Compound **21** was synthesized by following the general procedure E. Yield 94%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.82 (m, 4H), 7.21 (t, 2H, *J* = 7.32 Hz), 6.90 (t, 2H, *J* = 7.32 Hz), 6.81 (d, 1H, *J* = 7.89 Hz), 4.25 (d, 1H, *J* = 1.83 Hz), 3.93 (t, 2H, *J* = 5.49 Hz), 3.52 (dd, 1H, *J* = 9.15, 1.83 Hz), 3.25 (t, 2H, *J* = 5.49 Hz), 1.91 (m, 1H), 1.04 (d, 1H, *J* = 6.57 Hz), 0.98 (d, 1H, *J* = 6.78 Hz) ; HRMS (FAB) calcd for $C_{22}H_{29}N_2O_7S^+$ $[M+H]^+$: 423.1512, found: 423.1545.

4.3.17. (*S*)-*N*-(3,4-Dimethoxybenzyl)-4-(4-isobutyl-2,5-dioxoimidazolidin-1-yl)benzenesulfonamide (22).

Compound **22** was synthesized by following the general procedure F. Yield 69%, white

solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.88 (d, 2H, *J* = 8.97 Hz), 7.50 (d, 2H, *J* = 8.79 Hz), 6.69-6.80 (m, 3H), 4.28 (m, 1H), 4.04 (s, 2H), 3.76 (s, 3H), 3.71 (s, 3H), 1.61-1.95 (m, 3H), 1.01 (d, 6H, *J* = 6.42 Hz); HRMS (FAB) calcd for $C_{28}H_{32}N_3NaO_7S_2^+$ $[M+Na]^+$: 578.1859, found: 578.1909.

4.3.18. Benzyl (*S*)-(4-methyl-1-oxopentan-2-yl)carbamate (23).

Compound **23** was synthesized by following the reported procedures.[44] Yield 60% in three steps, yellow solid; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.58 (s, 1H), 7.30-7.36 (m, 5H), 5.15 (m, 1H), 5.10 (s, 2H), 4.33 (m, 1H), 1.63-1.80 (m, 2H), 1.35-1.44 (m, 1H), 0.95 (m, 6H); MS (ESI) *m/z* 521 $[2M+Na]^+$.

4.3.19. Benzyl (*S*)-(1-((4-(N-(3,4-dimethoxybenzyl)sulfamoyl)phenyl)amino)-4-methylpentan-2-yl)carbamate (24).

Compound **24** was synthesized by following the general procedure G. Yield 59%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.52 (m, 2H), 7.31-7.32 (m, 5H), 6.64-6.81 (m, 5H), 5.05 (d, 2H, *J* = 3.14 Hz) 3.92 (s, 2H), 3.87 (m, 1H), 3.76 (s, 3H), 3.72 (s, 3H), 3.17 (m, 2H), 1.70 (m, 1H), 1.28-1.44 (m, 2H), 0.91 (dd, 6H, *J* = 14.52, 6.78 Hz); MS (FAB) *m/z* 556 $[M+H]^+$.

4.3.20. (*S*)-4-((2-Amino-4-methylpentyl)amino)-N-(3,4-dimethoxybenzyl)benzenesulfonamide (25).

Compound **25** was synthesized by following the general procedure D. Yield 92%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.52 (m, 2H), 6.62-6.82 (m, 5H), 3.94 (s, 2H), 3.77

(s, 3H), 3.73 (s, 3H), 3.72 (m, 1H), 2.98-3.01 (m, 2H), 1.82 (m, 1H), 1.28-1.34 (m, 2H), 0.94 (dd, 6H, *J* = 12.52, 6.68 Hz); MS (ESI) *m/z* 422 $[M+H]^+$.

4.3.21. (*S*)-*N*-(3,4-Dimethoxybenzyl)-4-(4-isobutyl-2-oxoimidazolidin-1-yl)benzenesulfonamide (26).

Compound **26** was synthesized by following the general procedure F. Yield 51%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 7.68 (m, 4H), 6.77 (s, 1H), 6.73 (d, 1H, *J* = 1.83 Hz), 6.69 (m, 1H), 4.08 (t, 1H, *J* = 8.79 Hz), 4.00 (s, 2H), 3.92 (m, 1H), 3.77 (s, 3H), 3.71 (s, 3H), 3.53 (dd, 1H, *J* = 9.15, 6.42 Hz), 1.78 (m, 1H), 1.43-1.62 (m, 2H), 0.99 (d, 6H, *J* = 6.60 Hz); HRMS (FAB) calcd for $C_{22}H_{30}N_3O_5S^+$ $[M+H]^+$: 448.1828, found: 448.1871.

V. Part 4. Discovery of New Series of Simplified Leucyladenylate Sulfamates as a Novel LRS-targeted mTORC1 Inhibitors

1. Design background & strategy

Our group currently reported that (*S*)-2-hydroxy-4-methylpentanoyl adenylate sulfamate (**1**) as a potent mTORC1 inhibitor, which possesses selective anti-cancer activity against human colorectal cancer cells in which mTORC1 is hyperactive.[30] Furthermore, we discovered simplified leucyladenylate structures that can maintain the inhibitory activity of mTORC1 but also have more improved physicochemical properties to further develop as a pre-clinical candidate.[43]

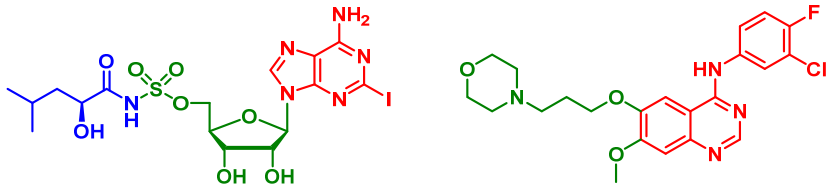


Figure 31. (*S*)-2-hydroxy-4-methylpentanoyl adenylate sulfamate (**1**) and gefitinib (**2**).

In our continuing efforts to develop LRS-targeted mTORC1 inhibitors as a potential anti-cancer agent and further expand our in-house library of simplified leucyladenylates analogues, we decided to design new scaffolds based on the gefitinib structure (**Figure.**

31). We devised new series of simplified structures by introducing *N*-(3-chloro-4-fluorophenyl) quinazolin-4-amine instead of adenine group and various linker structures to replace 5-*O*-sulfamoylribose (**Figure. 32**).

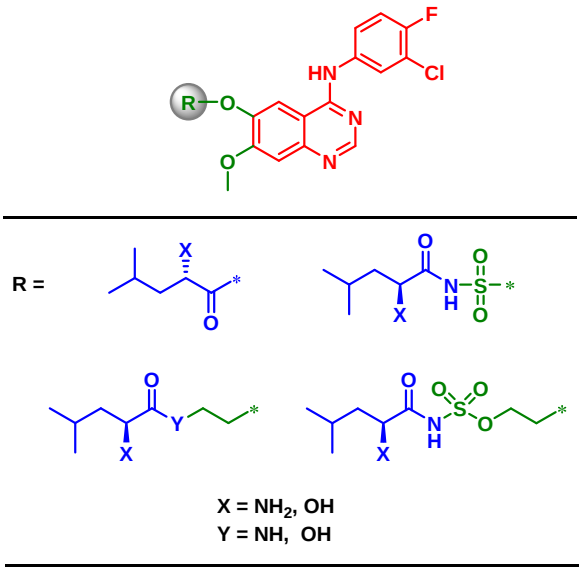


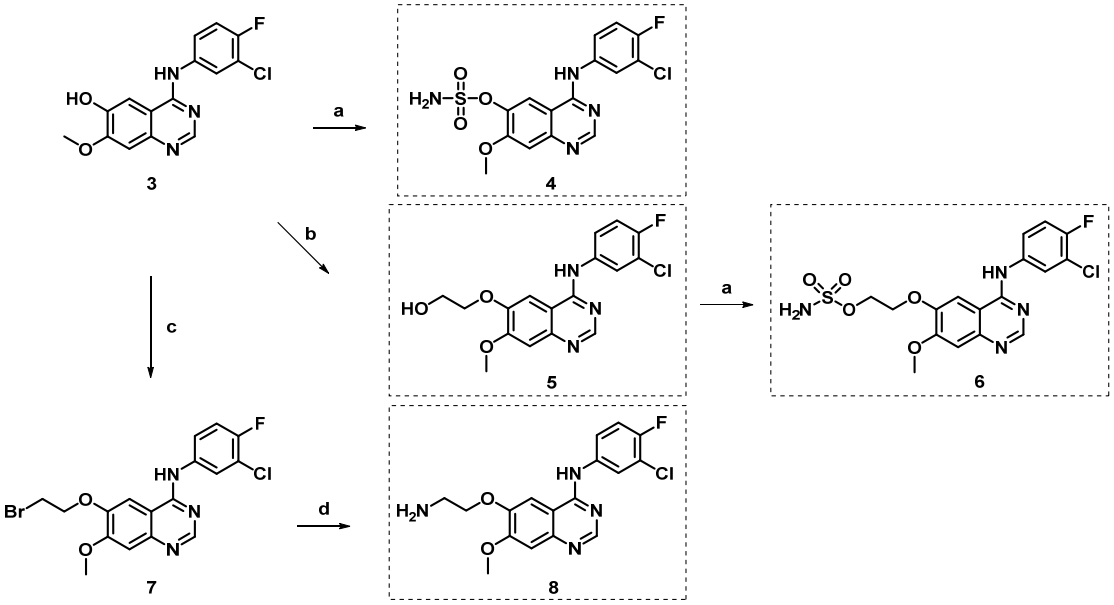
Figure 32. New simplified adenylates in conjunction with gefitinib structure.

2. Result and Discussion

2.1. Chemistry

Synthesis of intermediates (**4**, **5**, **6** and **8**) began with compound **3,** which was prepared by following the reported procedures (**scheme 23**).[45] To synthesize the intermediate **4**, compound **3** was reacted with sulfamoyl chloride prepared in a quantitative yield from chlorosulfonyl isocyanate according to previously reported procedures.[35] Ethyl alcohol linker was introduced under basic condition to give intermediate **5**, which was then

subsequently sulfamoylated to give intermediate **6**. *O*-alkylation of compound **3** with 1,2-dibromoethane gave compound **7**, which was then reacted with aqueous ammonia solution to produce primary amino group.

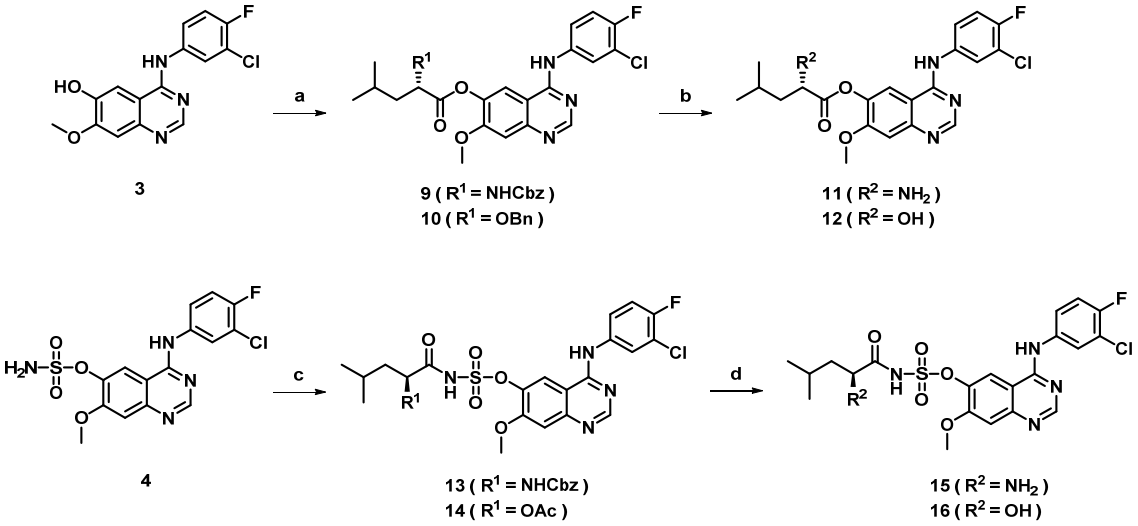


Scheme 23. Synthesis of intermediates from compound **3**.

Reagents & conditions: (a) Sulfamoyl chloride, DMA, 0 ^{o}C to r.t, overnight; (b) 2-Bromoethanol, K_2CO_3, DMF, r.t, 12 h; (c) 1,2-Dibromoethane, K_2CO_3, DMF, r.t, 12 h; (d) NH_4OH, DMF, 50 ^{o}C, 24 h.

Synthesis of compounds without ethyl linker in simplified adenylate structure (**11, 12, 15** and **16**) were carried out as illustrated in **Scheme 24**. Compound **3** was coupled with *N*-Cbz leucine and *O*-benzyl protected (2*S*)-hydroxyisocaproic acid (HICA, *l*-leucic acid) to afford compounds **9** and **10**, respectively. Also, intermediate **4** was condensed with *N*-Cbz leucine and *O*-acetyl protected HICA to give compounds **13** and **14**, respectively. *O*-

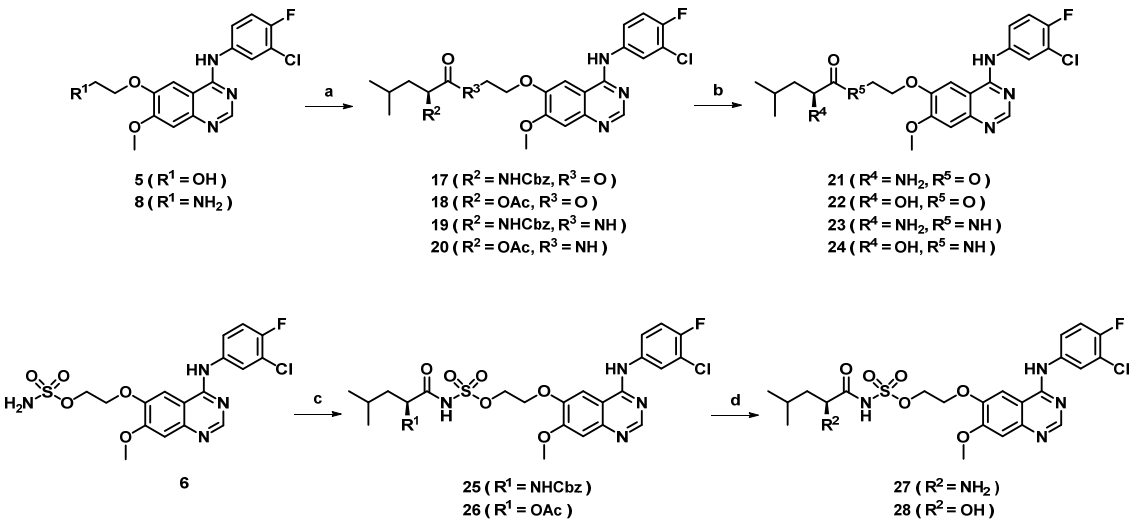
benzyl or O-acetyl protected HICA were prepared by benzylation or acetylation of commercially available *L*-leucic acid. Deprotection of the α-amino or α-hydroxyl group of compounds **9, 10, 13** and **14** provided the final compounds **11, 12, 15** and **16**, respectively.



Scheme 24. Synthesis of simplified leucyladenylate sulfamates analogues without ethyl linker.

Reagents & conditions: (a) Cyanuric chloride, TEA, Acetone, *N*-Cbz leucine for **9**, *O*-benzyl protected *L*-leucic acid for **10**, 0 ^{o}C to r.t, 2 h; (b) Pd/C, H_2, 2M NH_3 in MeOH, r.t, overnight for **11**, BBr_3, MC, -78 ^{o}C to r.t, 12 h for **12**; (c) DCC, DMAP, anhyd. MC, *N*-Cbz leucine for **13**, *O*-acetyl protected *L*-leucic acid for **14,** r.t, 2 h; (d) Pd/C, H_2, 2M NH_3 in MeOH, r.t, overnight for **15**, 0.02M NaOMe, 0 ^{o}C to r.t, 2 h for **16.**

Compounds containing ethyl linker in simplified adenylate structure (**21-24**, **27** and **28**) were synthesized as shown in **scheme 25**. Compounds **17-28** were synthesized by following the same procedures described in **scheme 24**.



Scheme 25. Synthesis of simplified leucyladenylate sulfamates analogues with ethyl linker.

Reagents & conditions: (a) Cyanuric chloride, TEA, Acetone, *N*-Cbz leucine for **17** and **19**, *O*-acetyl protected *L*-leucic acid for **18** and **20,** 0 ^{o}C to r.t, 2 h; (b) Pd/C, H_2, 2M NH_3 in MeOH, r.t, overnight for **21** and **23**, 0.02M NaOMe, 0 ^{o}C to r.t, 2 h for **22** and **24**; (c) DCC, DMAP, anhyd. MC, *N*-Cbz leucine for **25**, *O*-acetyl protected *L*-leucic acid for **26**, r.t, 2 h; (d) Pd/C, H_2, 2M NH_3 in MeOH, r.t, overnight for **27**, 0.02M NaOMe, 0 ^{o}C to r.t, 2 h for **28**.

2.2. Biological Activity

First, we examined the effects of synthesized compounds on cellular mTORC1 activation pathway by leucine-induced phosphorylation of S6Kinase (S6K) by immunoblotting method. In our previous studies, we have discovered that LRS-targeted inhibitors block the phosphorylation of S6K by inhibiting the mTORC1 pathway.[25,30,43] As a primary screening, we pretreated HEK293 cells with each final compounds at one fixed concentration (200 μM) as well as with rapamycin (100 nM) and leucinol (800 μM) as a

comparison, and then activated mTORC1 by treating the cells with leucine for 10 min. As shown in **Figure. 33**, pretreatment of rapamycin and leucinol blocked leucine-induced phosphorylation of S6K according to a weak pS6K band intensity. Among the synthesized compounds, **16**, **21**, **22** and **23** showed more potent inhibition of S6K phosphorylation than leucinol, while compounds **11**, **12**, **27** and **28** did not inhibit S6K phosphorylation, suggesting that the distance between the leucyl side chain and adenylate binding site is critical to activity. Compounds which showed mTORC1 inhibition (**16**, **21**, **22** and **23)** have sulfonamide, ethyl alcohol or ethyl amino linker in simplified adenylate structure. Specifically, in the case of compounds with sulfonamide linker, compound which has α-hydroxyl group (**16**) showed more potent inhibition than the α-amino group containing counterpart (**15**). However, in the case of compounds with ethyl amino linker, compound with α-amino group (**23**) showed more potent inhibition than the compound with α-hydroxyl group (**24**), suggesting that the binding interactions may change depending on the liker structures.

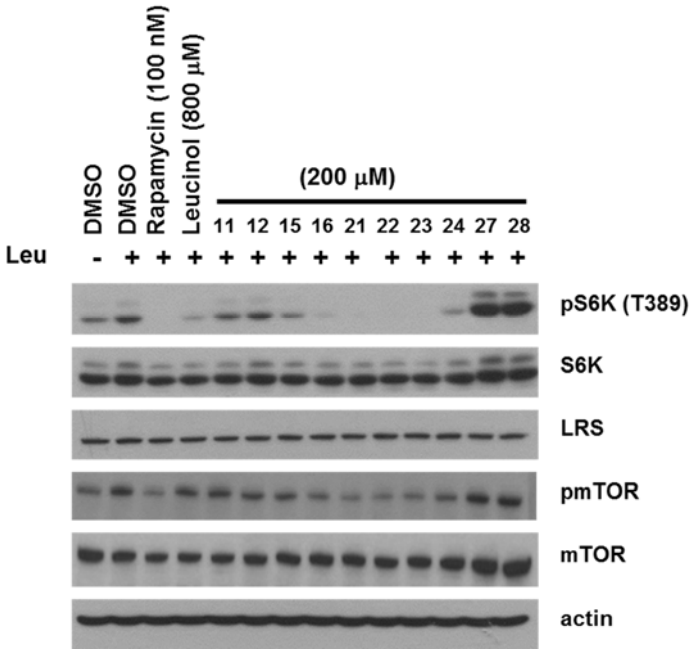


Figure 33. Inhibition of leucine-induced mTORC1 activation in HEK293 cells treated with compounds at fixed concentrations.

Next, we examined the dose-dependent inhibition of mTORC1 with compounds **16**, **21**, **22** and **23** based on the primary screening (**Figure. 34**). Interestingly, only compounds **16** and **22** appeared to inhibit S6K phosphorylation in a dose-dependent manner. Compounds **16**, **21** and **22** started to inhibit S6K phosphorylation strongly at 100 μM, showing a significant reduction in pS6K band intensities, but compound **23** did not inhibit S6K phosphorylation at the same concentration. When we examined expression level of pmTOR, the band intensities were not affected by the compounds, suggesting that these compounds were LRS-targeted mTORC1 inhibitors.

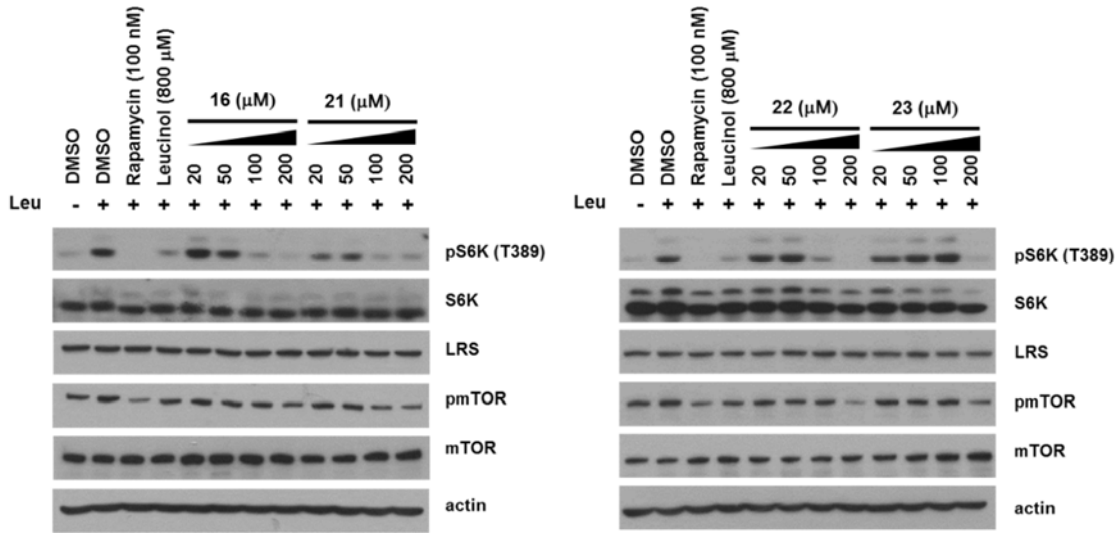


Figure 34. Dose-dependent inhibition of leucine-induced mTORC1 activation of compounds **16**, **21**, **22** and **23** in HEK293 cells.

To further investigate the inhibitory mechanism whether these simplified analogues affect catalytic activity of LRS or not, we performed aminoleucylation assays with compounds **16**, **21**, **22** and **23**. As shown in **Figure. 35**, all tested compounds did not inhibit catalytic function of LRS even at micromole range of concentration, indicating that these compounds selectively acted on LRS-mediated mTORC1 activation pathway without direct effect on catalytic activity of LRS. This result suggests that these simplified adenylate analogues selectively bind to LRS to inhibit mTORC1 pathway, which is in agreement with our previous report.[43]

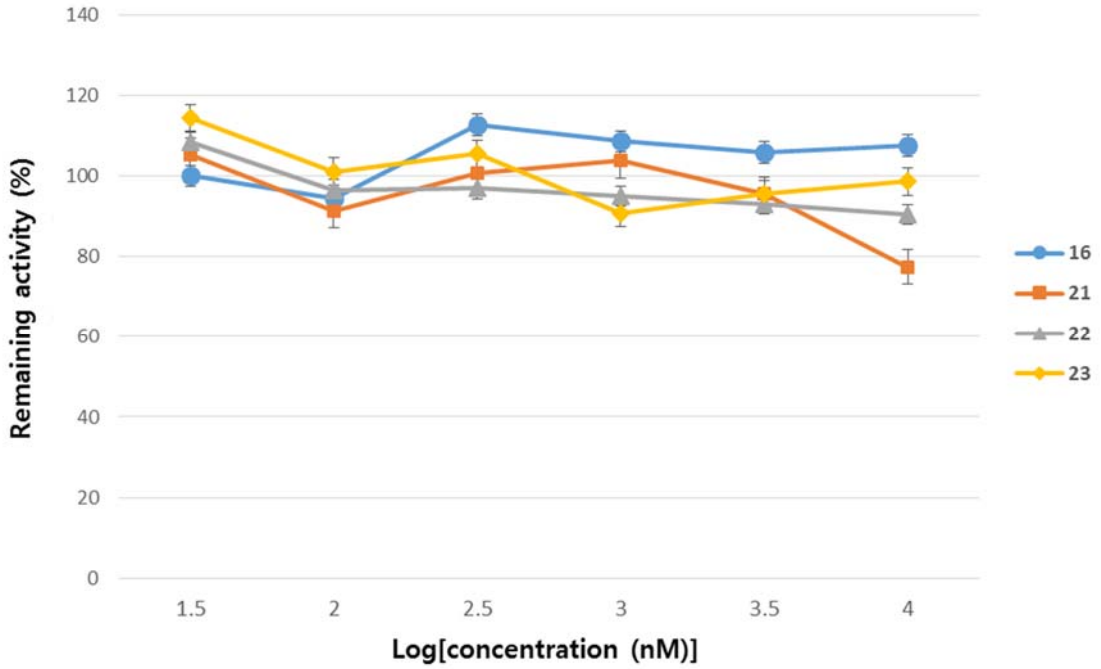


Figure 35. Inhibition of catalytic leucylation by compounds **16**, **21**, **22** and **23**.

Finally, to assess the anticancer activity of **16**, **21**, **22** and **23**, we performed the sulforhodamine B (SRB) colorimetric assays for cytotoxicity.[38] Compounds **16**, **21**, **22** and **23** were treated with six different types of cancer cell lines along with etoposide as a positive control. As shown in **Table 3**, all compounds showed moderate cytotoxicity with low micromole range against various cancer cell lines compared with etoposide.

Table 3. Relative Cell Growth Inhibition of compounds **16**, **21**, **22** and **23** for Various Cancer Cell Types.[a]

IC_{50} (μM)	A549	HCT116	K562	MDA-MB-231	SK-HEP-1	SNU638
16	4.38	4.04	4.10	6.23	3.30	4.94
21	3.59	3.97	4.01	3.65	1.94	3.86

22	3.16	3.12	3.89	2.05	1.39	3.41
23	2.79	4.24	3.83	4.59	2.21	2.97
Etoposide	0.30	1.06	0.76	1.53	0.63	1.05

[a]A549, lung cancer cells; HCT116, colon cancer cells; K562, leukemia cells; MDA-MB-231, breast cancer cells; SK-Hep-1, liver cancer cells; SNU638, stomach cancer cells; MRC5, lung normal epithelial cell.

3. Conclusion

We have developed a new series of simplified leucyladenylate sulfamate analogues that can directly bind to LRS to inhibit mTORC1 activation pathway. In this new series, we introduced *N*-(3-chloro-4-fluorophenyl) quinazolin-4-amine to replace the adenine group and various linker structures to replace 5-*O*-sulfamoylribose group that can maintain the activity but also improve the druggable physicochemical properties such as reduced polarity and asymmetric centers. Compounds with sulfonamide, ethyl alcohol or ethyl amino linker in simplified adenylate structure (**16**, **21**, **22** and **23)** showed potent inhibition than compounds with the no linker or sulfamoylated ethyl alcohol linker without affecting the catalytic activity of LRS. Furthermore, these compounds showed general cytotoxicity against various types of cancer cell lines, suggesting that they have a potential as effective anticancer agent.

4. Experimental

4.1. General Experimental

All chemical reagents were commercially available. Silica gel column chromatography was performed on Silica Gel 60, 230–400 mesh, Merck. Proton NMR spectra were

recorded on a JEOL JNM-LA 300 at 300 MHz and Bruker Analytik, DE/AVANCE Digital 400 at 400 MHz or Bruker AMX-500 (500 MHz) spectrometer. Chemical shifts are reported in ppm units with tetramethylsilane as a reference standard. Mass spectra and HRMS results were recorded on VG Trio-2 GC−MS instrument and JEOL JMS-AX instrument, respectively.

4.2. General procedure

4.2.1. Sulfamoylation (Procedure A)

To a solution of starting material in DMA (6 ml) was added freshly prepared sulfamoyl chloride (1.5 equiv.) at 0^{o}C and gradually increased to room temperature. The reaction mixture was stirred for overnight until the starting material disappeared and quenched with MeOH (6 ml) at 0^{o}C. The reaction mixture evaporated and purified by silica gel flash column chromatography to afford the corresponding sulfamoylated product.

4.2.2. *O*-alkylation (Procedure B)

To a solution of starting material in DMF (6 ml) was added K_2CO_3 (3 equiv.) followed by 2-bromoethanol (3 equiv.) or 1,2-dibromoethane (3 equiv.). The reaction mixture was stirred at room temperature for 12 h and quenched with water (10 ml). The mixture was extracted with ethyl acetate (2 x 50 ml), washed with brine (3 x 50 ml), dried over $MgSO_4$ and evaporated. The residue was purified by silica gel flash column chromatography to afford the corresponding *O*-alkylated product.

4.3. Procedures and Chemical spectra

4.3.1. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl sulfamate (4)

Compound **4** was prepared by following the general procedure A. Yield 75%, white solid; ^{1}H-NMR (300 MHz, DMSO) δ 8.88 (s, 1H), 8.56 (s, 1H), 7.95 (dd, 1H, *J* = 6.78, 2.22 Hz), 7.63 (m, 1H), 7.54 (t, 1H, *J* = 8.97 Hz), 7.25 (s, 1H), 3.99 (s, 3H).

4.3.2. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethan-1-ol (5)

Compound **5** was prepared by following the general procedure B. Yield 30%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.42 (s, 1H), 8.04 (dd, 1H, *J* = 6.60, 2.58 Hz), 7.74 (s, 1H), 7.69-7.63 (m, 1H), 7.25 (t, 1H, *J* = 8.97 Hz), 7.18 (s, 1H), 4.48 (t, 1H, *J* = 6.06 Hz), 3.99 (s, 3H), 3.71 (t, 1H, *J* = 6.03 Hz).

4.3.3. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl sulfamate (6)

Compound **6** was prepared by following the general procedure A. Yield 75%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.91 (dd, 1H, *J* = 6.57, 2.55 Hz), 7.65 (s, 1H), 7.60-7.56 (m, 1H), 7.16 (t, 1H, *J* = 8.97 Hz), 7.09 (s, 1H), 4.49-4.46 (m, 2H), 4.38-4.35 (m, 2H), 3.91 (s, 3H).

4.3.4. 6-(2-bromoethoxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxyquinazolin-4-amine (7)

Compound **7** was prepared by following the general procedure B. Yield 45%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.00 (dd, 1H, *J* = 6.60, 2.58 Hz), 7.74 (s, 1H), 7.69-7.63 (m, 1H), 7.25 (t, 1H, *J* = 8.97 Hz), 7.18 (s, 1H), 4.50 (t, 1H, *J* = 6.06 Hz), 4.00 (s, 3H), 3.81 (t, 1H, *J* = 6.03 Hz).

4.3.5. 6-(2-aminoethoxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxyquinazolin-4-amine (8)

To a solution of compound **7** in DMF (6 ml) was added NH_4OH (excess amount) at room temperature and gradually increased to 50^{o}C. The reaction mixture was stirred for 24 h until the starting material disappeared and DMF was removed under reduced pressure to afford the primary amine. The crude product was directly used to next reaction. Yield 94%, yellow oil; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.41 (s, 1H), 8.14 (s, 1H), 8.01-7.99 (m, 1H), 7.71-7.65 (m, 1H), 7.24 (t, 1H, *J* = 8.97 Hz), 7.16 (s, 1H), 4.35-4.24 (m, 4H), 3.99 (s, 3H).

4.3.6. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl ((benzyloxy)carbonyl)-*L*-leucinate (9)

Compound **9** was prepared by following the procedure in previous report.[43] Yield 32%,

white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.51 (s, 1H), 8.03 (s, 1H), 8.01 (m, 1H), 7.66-7.63 (m, 1H), 7.37-7.20 (m, 7H), 5.14 (s, 2H), 4.55 (m, 1H), 3.92 (s, 3H), 1.85 (m, 2H), 1.03 (t, 1H, *J* = 6.24 Hz).

4.3.7. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (*S*)-2-(benzyloxy)-4-methylpentanoate (10)

Compound **10** was prepared by following the procedure in previous report.[43] Yield 58%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.54 (s, 1H), 8.17 (s, 1H), 8.02 (m, 1H), 7.70-7.65 (m, 1H), 7.41-7.22 (m, 7H), 4.55 (d, 1H, *J* = 11.70 Hz), 4.33 (m, 1H), 4.01 (s, 3H), 1.88 (m, 1H), 1.23 (t, 1H, *J* = 7.14 Hz), 0.99 (d, 3H, *J* = 6.42 Hz), 0.91 (d, 3H, *J* = 6.39 Hz).

4.3.8. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl *L*-leucinate (11)

Compound **11** was prepared by following the procedure in previous report.[43] Yield 58%, white solid; ^{1}H-NMR (300 MHz, DMSO) δ 9.68 (s, 2H), 9.47 (s, 1H), 8.46 (s, 1H), 8.20 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.83 (m, 1H), 7.77 (s, 1H), 7.40 (t, 1H, *J* = 8.97 Hz), 7.20 (s, 1H), 3.96 (s, 3H), 3.60 (m, 1H), 1.44 (m, 1H), 1.22 (s, 2H), 0.89-0.82 (m, 6H); MS (FAB) m/z 433 (M+H).

4.3.9. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (*S*)-2-hydroxy-4-methylpentanoate (12)

BBr_3 (1.5 equiv.) was added dropwise to a stirred solution of compound **10** (1 equiv.) in CH_2Cl_2 at -78°C and gradually increased to room temperature. The reaction mixture was stirred for 12 h until the starting material disappeared and quenched with MeOH at 0°C. The reaction mixture evaporated and purified by silica gel flash column chromatography to afford compound **12.** Yield 60%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.02 (s, 1H), 7.92 (dd, 1H, *J* = 6.60, 2.58 Hz), 7.57 (m, 1H), 7.20 (s, 1H), 7.16 (t, 1H, *J* = 8.97 Hz), 4.41 (m, 1H), 3.88 (s, 3H), 1.91 (m, 1H), 1.73-1.68 (m, 2H), 0.94 (dd, 6H, *J* = 6.75, 2.73 Hz); MS (FAB) m/z 434 (M+H).

4.3.10. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (((benzyloxy)carbonyl)-*L*-leucyl)sulfamate (13)

Compound **13** was prepared by following the procedure in previous report.[32] Yield 45%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.19 (s, 1H), 7.95 (dd, 1H, *J* = 6.78, 2.55 Hz), 7.20 (s, 1H), 7.17-7.13 (m, 6H), 4.90 (d, 2H, *J* = 4.02 Hz), 4.15-4.08 (m, 1H), 3.98 (s, 3H), 1.72 (m, 1H), 1.56-1.49 (m, 2H), 0.99 (d, 6H, *J* = 6.57 Hz).

4.3.11. (*S*)-1-((((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)sulfonyl)amino)-4-methyl-1-oxopentan-2-yl acetate (14)

Compound **14** was prepared by following the procedure in previous report.[32] Yield 49%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.50 (s, 1H), 8.14 (s, 1H), 8.00 (dd, 1H, *J* = 6.78, 2.76 Hz), 7.71-7.67 (m, 1H), 7.25 (t, 1H, *J* = 8.97 Hz), 7.24 (s, 1H), 4.00 (s, 3H),

2.09 (s, 3H), 1.70-1.58 (m, 3H), 0.90 (dd, 6H, *J* = 6.60, 4.95 Hz).

4.3.12. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (*L*-leucyl)sulfamate (15)

Compound **15** was prepared by following the procedure in previous report.[43] Yield 52%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.34 (s, 1H), 7.69-7.64 (m, 3H), 7.16 (s, 1H), 7.12 (t, 1H, *J* = 8.61 Hz), 4.04 (s, 3H), 3.98 (dd, 1H, *J* = 10.44, 3.12 Hz), 1.85-1.75 (m, 1H), 1.70-1.46 (m, 2H), 0.93 (t, 6H, *J* = 6.57 Hz); MS (FAB) m/z 512 (M+H).

4.3.13. 4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl (*S*)-(2-hydroxy-4-methylpentanoyl)sulfamate (16)

Compound **16** was prepared by following the procedure in previous report.[43] Yield 46%, white solid; ^{1}H-NMR (300 MHz, DMSO) δ 9.60 (s, 1H), 9.46 (s, 2H), 8.46 (s, 1H), 8.20 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.85-7.79 (m, 1H), 7.76 (s, 1H), 7.40 (t, 1H, *J* = 9.18 Hz), 7.20 (s, 1H), 3.96 (s, 3H), 3.15 (d, 1H, *J* = 4.38 Hz), 1.98 (m, 1H), 1.75-1.55 (m, 2H), 0.93 (m, 6H); MS (FAB) m/z 513 (M+H).

4.3.14. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl ((benzyloxy)carbonyl)-*L*-leucinate (17)

Compound **17** was prepared by following the procedure in previous report.[43] Yield 37%,

white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.01 (dd, 1H, *J* = 6.75, 2.73 Hz), 7.75 (s, 1H), 7.68 (m, 1H), 7.27-7.16 (m, 7H), 5.03 (s, 2H), 4.60-4.40 (m, 4H), 4.23 (t, 1H, *J* = 6.96 Hz), 3.97 (s, 3H), 1.68 (m, 1H), 1.59-1.54 (m, 2H), 0.88 (d, 3H, *J* = 6.39 Hz), 0.85 (d, 3H, *J* = 6.39 Hz).

4.3.15. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (*S*)-2-acetoxy-4-methylpentanoate (18)

Compound **18** was prepared by following the procedure in previous report.[43] Yield 43%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.45 (s, 1H), 8.00 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.76 (s, 1H), 7.69-7.64 (m, 1H), 7.25 (t, 1H, *J* = 8.97 Hz), 7.18 (s, 1H), 5.02-4.95 (m, 2H), 4.64-4.50 (m, 2H), 4.42 (m, 1H), 4.00 (s, 3H), 2.06 (s, 3H), 1.79-1.57 (m, 3H), 0.96-0.86 (m, 6H).

4.3.16. benzyl (*S*)-(1-((2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)amino)-4-methyl-1-oxopentan-2-yl)carbamate (19)

Compound **19** was prepared by following the procedure in previous report.[43] Yield 33%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.46 (s, 1H), 8.05 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.79 (s, 1H), 7.68 (m, 1H), 7.28-7.16 (m, 7H), 5.02 (s, 2H), 4.24 (m, 2H), 4.13 (m, 1H), 3.98 (s, 3H), 3.69 (m, 2H), 1.79-1.57 (m, 3H), 0.91 (t, 6H, *J* = 6.39 Hz).

4.3.17. (*S*)-1-((2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)amino)-4-methyl-1-oxopentan-2-yl acetate (20)

Compound **20** was prepared by following the procedure in previous report.[43] Yield 28%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.96 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.71 (s, 1H), 7.61 (m, 1H), 7.16 (t, 1H, *J* = 8.97 Hz), 7.10 (s, 1H), 4.92 (m, 1H), 4.17 (m, 2H), 3.92 (s, 3H), 3.59 (m, 2H), 2.00 (s, 3H), 1.64 (m, 1H), 1.48 (m, 2H), 0.82 (t, 6H, *J* = 6.42 Hz).

4.3.18. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl *L*-leucinate (21)

Compound **21** was prepared by following the procedure in previous report.[43] Yield 54%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.00 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.75 (s, 1H), 7.69-7.66 (m, 1H), 7.26 (t, 1H, *J* = 6.42 Hz), 7.19 (s, 1H), 4.26 (m, 2H), 4.00 (s, 3H), 3.99 (m, 2H), 3.69 (m, 1H), 1.75-1.50 (m, 3H), 0.92 (t, 6H, *J* = 6.21 Hz); MS (FAB) m/z 477 (M+H).

4.3.19. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (*S*)-2-hydroxy-4-methylpentanoate (22)

Compound **22** was prepared by following the procedure in previous report.[43] Yield 54%, white solid; ^{1}H-NMR (300 MHz, DMSO) δ 11.05 (s, 1H), 8.81 (s, 1H), 8.20 (s, 1H), 8.03 (m, 1H), 7.73 (m, 1H), 7.65 (t, 1H, *J* = 8.79 Hz), 7.31 (s, 1H), 4.24 (m, 2H), 4.00 (s, 3H),

3.84 (m, 2H), 2.18 (m, 1H), 1.76 (m, 2H), 0.87 (m, 6H); MS (FAB) m/z 478 (M+H).

4.3. 20. (*S*)-2-amino-*N*-(2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)-4-methylpentanamide (23)

Compound **23** was prepared by following the procedure in previous report.[43] Yield 55%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.49 (s, 1H), 8.12 (s, 1H), 7.99 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.75 (s, 1H), 7.71-7.66 (m, 1H), 7.25 (t, 1H, *J* = 8.97 Hz), 7.16 (s, 1H), 4.25 (m, 2H), 3.98 (s, 3H), 3.80 (m, 1H), 3.73 (m, 2H), 1.75-1.58 (m, 3H), 0.90 (dd, 6H, *J* = 6.21, 5.31 Hz); MS (FAB) m/z 476 (M+H).

4.3.21. (*S*)-*N*-(2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl)-2-hydroxy-4-methylpentanamide (24)

Compound **24** was prepared by following the procedure in previous report.[43] Yield 57%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.36 (s, 1H), 7.94 (d, *J* = 6.96 Hz), 7.73 (s, 1H), 7.60 (m, 1H), 7.15 (t, 1H, *J* = 8.97 Hz), 7.09 (s, 1H), 4.18 (t, 2H, *J* = 5.49 Hz), 3.97 (m, 1H), 3.92 (s, 3H), 3.62 (m, 2H), 1.75 (m, 1H), 1.37-1.45 (m, 2H), 0.82 (dd, 6H, *J* = 6.68, 4.05 Hz); MS (FAB) m/z 477 (M+H).

4.3.22. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (((benzyloxy)carbonyl)-*L*-leucyl)sulfamate (25)

Compound **25** was prepared by following the procedure in previous report.[32] Yield 46%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.43 (s, 1H), 8.01 (dd, 1H, *J* = 6.78, 2.58 Hz), 7.91 (s, 1H), 7.67 (m, 1H), 7.20-7.11 (m, 7H), 4.94 (s, 2H), 4.41-4.37 (m, 4H), 4.08 (m, 1H), 3.96 (s, 3H), 1.75-1.45 (m, 3H), 0.86 (m, 6H).

4.3.23. (*S*)-1-(((2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethoxy)sulfonyl)amino)-4-methyl-1-oxopentan-2-yl acetate (26)

Compound **26** was prepared by following the procedure in previous report.[32] Yield 58%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.41 (s, 1H), 8.04 (dd, 1H, *J* = 6.78, 2.76 Hz), 7.92 (s, 1H), 7.76-7.71 (m, 1H), 7.17 (t, 1H, *J* = 8.97 Hz), 7.10 (s, 1H), 4.83-4.79 (m, 1H), 4.44 (m, 4H), 3.98 (s, 3H), 1.74-1.55 (m, 3H), 0.87 (dd, 6H, *J* = 9.54, 6.60 Hz).

4.3.24. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (*L*-leucyl)sulfamate (27)

Compound **27** was prepared by following the procedure in previous report.[43] Yield 65%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.44 (s, 1H), 8.06 (dd, 1H, *J* = 6.77, 2.76 Hz), 7.89 (s, 1H), 7.73 (m, 1H), 7.22 (t, 1H *J* = 8.97 Hz), 7.14 (s, 1H), 4.48 (m, 4H), 3.98 (s, 3H), 3.60 (m, 1H), 1.56-1.75 (m, 3H), 0.91 (m, 6H); MS (FAB) m/z 556 (M+H).

4.3.25. 2-((4-((3-chloro-4-fluorophenyl)amino)-7-methoxyquinazolin-6-yl)oxy)ethyl (*S*)-(2-hydroxy-4-methylpentanoyl)sulfamate (28)

Compound **28** was prepared by following the procedure in previous report.[43] Yield 77%, white solid; ^{1}H-NMR (300 MHz, CD_3OD) δ 8.43 (s, 1H), 8.06 (dd, 1H, J = 6.78, 2.76 Hz), 7.90 (s, 1H), 7.70 (m, 1H), 7.20 (t, 1H, J = 9.15 Hz), 7.12 (s, 1H), 4.80 (m, 1H), 4.47 (m, 4H), 3.98 (s, 3H), 1.80 (m, 1H), 1.55-1.42 (m, 2H), 0.86 (dd, 6H, J = 6.60, 1.65 Hz); MS (FAB) m/z 557 (M+H).

VI. References

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박사학위논문 국문초록

인간 단백질합성효소 Leucyl-tRNA Sythetase (LRS)를 표적으로 하는 신규 Mammalian Target of Rapamycin Complex 1 (mTORC1) 저해제 개발

Mammalian target of rapamycin (mTOR)은 serine/threonine 단백질 인산화 효소로서 세포의 대사, 성장, 분열과 자가소화 작용을 조절하는 역할을 한다. 생체 내에서 mTOR는 mTOR complex 1 (mTORC1)과 mTOR complex 2 (mTORC2)의 두 개의 구조적으로, 기능성으로 다른 특성을 가진 단백질 복합체로 존재한다. Rapamycin에 sensitive하다고 알려진 mTORC1은 세포 내의 아미노산의 농도, 스트레스 레벨, 세포 증식인자, 에너지 상태 등 세포 안과 밖의 다양한 신호들을 감지하여 단백질의 합성을 조절한다. mTORC1이 활성화가 되면 세포 신호 전달의 하위 기질인 S6 kinase 1 (S6K1)과 eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1)을 인산화 시킴으로써 신호 전달이 일어나고, 이 두 기질은 세포의 성장과 악성종양 형성에 중요한 역할을 한다고 알려져 있다.

Rapamycin은 mTORC1의 선택적인 저해제로서 잘 알려져 있으며, FK506-binding protein 12 (FKBP12)에 결합하여 복합체를 이루어 mTORC1의 FKBP12-rapamycin binding (FRB) domain에 결합하며, 이와 같은 단백질-단백질 간 결합을 통해 mTORC1의 효소 촉매 작용을 억제한다. S6K1과 4E-BP1을 포함하는 신호 전달 과정이 많은 조직에서 악성 종양 형성에 관여하고 있기 때문에 항암제로서 rapamycin과 그 유도체들에 대한 많은 연구가 진행되고 있다. Temsirolimus와 Everolimus는 발전적 콩팥 세포 암에 대한 치료제로 FDA의 승인을 받은 바 있으며, Ridaforolimus는 발전적 연조직 육종과 골육종에 대하여 최근 임상 시험 결과 좋은 치료효과를 보이는 것으로 보고되었다. 하지만, rapamycin과 그 유도체들의 선택적이고 강력한 효능에도 불구하고 여러 암환자를 대상으로 한 치료에서 일정한 결과를 얻지 못하였다. 그 이유로는 세포 독성을 갖는 억제제

가 아닌 세포 증식 억제제라는 점과 mTORC1의 신호전달을 일부만 저해시킨다는 제한 점이 있다. 따라서 mTORC1의 활성을 조절할 수 있는 또 다른 조절 인자를 표적으로 하는 억제제의 개발이 시급한 상황이며, 이는 rapamycin에 저항성을 갖는 암세포에 대하여 효과적인 새로운 항암 치료 전략이 될 수 있을 것이라 기대하였다.

mTORC1의 활성에 영향을 미치는 다양한 환경적 요인들 중, 단백질의 합성에 관여하는 필수 아미노산 중 류신이 아미노산에 의한 mTORC1의 활성화에 주된 조절 인자로 작용한다고 알려져 있다. 류신이 mTORC1을 활성화시키는 기전에 대하여 완벽히 규명되지는 않았지만, 최근 여러 연구 결과에 따르면 leucyl-tRNA synthetase (LRS)가 세포 안의 아미노산에 의해 mTORC1이 활성화되는 신호 전달 과정에 주요 인자인 RagD GTPase와 직접적으로 결합하여 류신의 센서로서 작용한다고 보고되었다. LRS는 세포 안에서 단백질 합성 효소로서 ATP 의존적으로 류신을 그 cognate transfer RNA (tRNA)에 연결시켜주는 반응을 촉매 한다. 또한, LRS에 류신이 결합하게 되면 RagD GTPase에 대하여 GTPase-activating protein (GAP)의 역할을 하는 것으로 보고 되었으며, RagD-GTP의 가수분해를 촉진하여 mTORC1을 활성화 시킨다. 또한, 류신에 의해 활성화된 mTORC1은 그 유도체인 류시놀에 의해 억제될 수 있다고 보고 된 바 있으며, 이 과정에서 LRS의 tRNA에 류신을 charging하는 활성에는 영향을 미치지 않는 것으로 보고 되었다.

선행 연구를 기반으로 하여 본 연구실에서는 첫 번째로, leucine의 유도체인 leucinol의 유도체들을 합성하여 LRS를 통해 활성화된 mTORC1을 저해하는지 여부를 확인하였고, 그 결과 mTORC1에 대하여 가장 강력한 활성을 보인 (S)-Isobutyloxazolidin-2-one의 화합물을 도출하였다. 이 화합물은 rapamycin에 저항성을 갖는 SW620 세포에 대하여 농도 의존적인 세포독성을 갖는 것을 확인하였다.

이후 LRS의 저해제로서 잘 알려진 leucyladenylate sulfamate 구조를 기반으로 하여 LRS를 표적으로 하는 mTORC1 저해제를 디자인하였다. 기존 저분자 유도체들보다 추가적인 binding interaction을 통해 좀 더 강력한 mTORC1 저해제 개발을 할 수 있을 것이라 기대하였다. 연구 결과, leucyladenylate sulfamate 구조에서

leucine의 a위치에 amino 그룹을 hydroxyl 그룹으로 치환하고, adenine의 2번 위치를 iodine으로 치환한 화합물이 농도 의존적으로 mTORC1을 억제하며, mTORC1이 과발현 되어있는 대장암 세포 (HCT116) 에서 선택적인 세포독성을 갖는 것을 확인하였다.

다음으로 이러한 유도체들이 화학적으로 극성이 높아 대량 합성상의 어려움이 있고, 낮은 생체 이용률로 인한 전임상 단계 진입의 어려움을 극복하고자 구조를 단순화하여 물리화학적 특성을 개선한 유도체들을 디자인하였다. 구조적으로 5-O-sulfamoylribose를 simplify하여 benzene sulfonamide 그룹으로 치환하였다. 본 연구실에서 보유하고 있는 화합물 라이브러리 중 adenine의 구조를 3,4-dimethoxyphenyl 또는 2-phenoxy 구조로 치환하여 화합물을 디자인하였고, side chain에는 선행 연구에서 좋은 결과를 보였던 rigid한 구조를 도입하여 구조 활성 연구를 진행하였다. 연구 결과, 5-O-sulfamoylribose를 benzenesulfonamide로 치환하고 adenine을 3,4-dimethoxyphenyl 그룹으로 치환한 유도체가 좋은 활성을 보였으며, 이들 화합물 중 선도 화합물 대비 3배 이상 더 강력한 활성을 보이는 화합물을 도출할 수 있었다. 또한, 이 화합물 역시 여섯 가지의 암세포에 대하여 전반적인 세포독성을 확인하였고 이러한 연구 결과로 LRS를 표적으로 하여 mTORC1의 활성을 저해하는 항암제 개발의 가능성을 확인하였다.

또한, 본 연구실의 simplified leucyladenylate 유도체 라이브러리 중, gefitinib 구조를 도입하여 새로운 scaffold를 갖는 화합물을 디자인하여 구조 활성 연구를 수행하였다. Adenine 구조를 N-(3-chloro-4-fluorophenyl)quinazolin-4-amine로 치환하였고, 5-O-sulfamoylribose 구조 대신에 다양한 linker를 갖는 화합물을 디자인하여 최적의 LRS을 표적으로 하는 mTORC1 저해제를 도출하고자 하였다. 연구 결과, leucyl side chain과 adenylate binding site 사이에 sulfonamide, ethyl alcohol 또는 ethyl amino linker를 포함하는 화합물에서 강력한 저해 활성을 확인하였다. linker의 길이가 너무 짧거나 긴 경우 LRS의 binding site에 화합물이 결합하지 못해 활성이 현저하게 감소하는 것을 확인할 수 있었으며, 이 화합물들 모두 여섯 가지의 암세포에 대하여 전반적인 세포독성을 확인할 수 있었다.

주 요 어: Leucyl-tRNA Synthetase, mTORC1 Inhibitor, Leucinol, Anticancer Agent